REMARKS

Claims 1-2, 4-16 are currently pending in the application. Applicant gratefully acknowledges the withdrawal of all rejections and objections as set forth in the previous Office Action. Moreover, Applicant gratefully acknowledges that the Office has determined that Claims 1 and 4-8 are free of the art.

Priority

Applicant gratefully acknowledges that priority has been extended to 60/176,515 for claims 1, 2, and 4-11. However, the Office Action asserts that claims 12-16 have an effective priority date of 1/12/2001. Applicant respectfully disagrees, and asserts that the priority date of the invention as presently claimed is January 12, 2000, the filing date of the '515 application. However, the priority date is not relevant to any of the outstanding rejections of these claims, and as such, Applicant respectfully requests the Office to withdraw its priority determination as moot.

Discussion of the 35 U.S.C. § 112 Rejection

Claims 9-16 stand rejected under 35 U.S.C. § 112 as failing to comply with the written description requirement because allegedly the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Office Action alleges that even though the originally filed application contemplates the chemotherapeutic agent of doxorubicin, there is no disclosure that doxorubicin is a topoisomerase inhibitor, or that the invention encompasses topoisomerase inhibitors as a

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class of compounds. The Office Action further alleges that the prior art teaches that doxorubicin is an anti-tumor antibiotic and that the mechanism of action is one of inhibiting DNA dependant RNA synthesis to delay or inhibit mitosis, citing *Cada*, which according to the Office Action is not commensurate in scope with a topoisomerase inhibitor. Applicants respectfully traverse this rejection.

The fundamental factual inquiry to determine whether a newly added claim complies with the written description requirement is "whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed." M.P.E.P. 2163(I)(B). Contrary to the Office Action's assertions, one skilled in the art as of the priority date of the pending claims would have understood that doxorubicin is a topoisomerase inhibitor. In fact, a search of Pubmed (www.ncbi.nlm.nih.gov/pubmed/) for "doxorubicin" and "topoisomerase inhibitor" revels over 100 articles published prior to the present invention's priority date. For Example, *Fink, et al.*, Clin. Can. Res., 4:1 (1998), attached as Exhibit A, describes the loss of DNA mismatch repair, and its affect on the resistance to chemotherapeutic agents. In this reference, Fink specifically describes doxorubicin as a topoisomerase II inhibitor. *See Fink, abstract* ("MMR-deficient cells have been reported to be resistant to the methylating agents procarbazine and temozolomide, the alkylatng agent busulfan, the platinum-containing drugs ciplatin and carboplatin, the antimetabolite 6-thioguanine, and the topoisomerase II inhibitors etoposide and doxorubicin."). In addition, *Mo, et al.*, Mol. Pharm., 55:216 (1999), attached as Exhibit B, in reporting that DNA damages signals induce Fas Ligand, described "[s]everal well-characterized Topo [topoisomerase] II inhibitors including doxorubicin, teniposide (VM-26), and etoposide (VP-

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Serial No.: 09/760,119
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16)." *See Mo* at 216, col. 2. As a further example, Harris, *et al.*, Clin. Can. Res., 4:1005 (1998), attached as Exhibit C, reports the induction of sensitivity to doxorubicin and etoposide by transfection of breast cancer cells with Heregulin ß-2. Harris also acknowledged that those skilled in the art would have understood that doxorubicin is a topoisomerase inhibitor. *See Harris*, abstract ("To study the relationship between chemotherapy response and activation of *HER2*, MCF-7 cells expressing biologically active Heregulin were assessed for response to doxorubicin and etoposide, both of which are topoisomerase IIα (topo IIα) inhibitors."). Applicant respectfully submits that these references, all published before the instant priority date, clearly establish that those skilled in the art as of the filing date of the present application, would have understood doxorubicin to be a topoisomerase II inhibitor.

Moreover, the Cada reference, cited in the Office Action, does not discount doxorubicin's role as a topoisomerase II inhibitor, and in fact Cada's description of doxorubicin is consistent with its function as a topoisomerase II inhibitor. Cada describes doxorubicin as an antibiotic-type agent, whose primary mechanism of action are to inhibit DNA-dependant RNA synthesis and to delay or inhibit mitosis. In comparison, Mo *et al.*'s description of the action of topoisomerase II inhibitors, including doxorubicin, is almost identical – "A consequence of [topoisomerase II inhibitor] drug actions is interference with transcription, DNA synthesis, and mitosis, eventually leading to cell death by apoptosis." Thus, Applicant respectfully submits the Cada reference is not relevant to the analysis of whether one skilled in the art would have understood doxorubicin to be a topoisomerase II inhibitor, and in fact, if anything, it supports that proposition.

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Furthermore, the application as filed does contemplate the use of topoisomerase II inhibitors, as exemplified by doxorubicin, in the present invention. For example, the application, at page 3, lines 15-18, explains that "[r]ecent studies have shown that a varity of DNA-damaging agents, including X-ray irradiation and several chemotherapeutic drugs (*e.g.*, alkylating agents and topoisomerase II inhibitors) cause necrosis or initiate pathways leading to apoptosis." Of course, Applicant understood that doxorubicin was a well-characterized topoisomerase II inhibitor, *see Mo* supra, and therefore the relationship between doxorubicin and topoisomerase II inhibitor did not need to be made explicit in order to convey possession to one skilled in the art.

Accordingly, Applicant respectfully submits that the specification conveys with reasonable clarity to one skilled in the art that the applicant was in possession of the invention, and requests withdrawal of this rejection and requests reconsideration of the claims.

Conclusion:

If the Examiner believes that a telephone or personal interview would expedite prosecution of the instant application, the Examiner is invited to call the undersigned at (312) 913-0001.

Respectfully submitted,

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Date: September 24, 2008

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EXHIBIT A

Minireview

The Role of DNA Mismatch Repair in Drug Resistance[1]

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Abstract

Loss of DNA mismatch repair (MMR) has been observed in a variety of human cancers. In addition to predisposing to oncogenesis, loss of MMR activity is of concern with respect to the use of chemotherapeutic agents to treat established tumors. Loss of MMR results in drug resistance directly by impairing the ability of the cell to detect DNA damage and activate apoptosis and indirectly by increasing the mutation rate throughout the genome. The MMR proteins are involved in mediating the activation of cell cycle checkpoints and apoptosis in response to DNA damage. MMR-deficient cells have been reported to be resistant to the methylating agents procarbazine and temozolomide, the alkylating agent busulfan, the platinum-containing drugs cisplatin and carboplatin, the antimetabolite 6-thioguanine, and the topoisomerase II inhibitors etoposide and doxorubicin. In the case of cisplatin, busulfan, temozolomide, and procarbazine, the degree of resistance has been shown to be sufficient to produce a large difference in clinical responsiveness *in vivo* in tumor model systems. The available preclinical data suggest that tumors that contain a significant fraction of cells deficient in MMR will demonstrate reduced responsiveness to specific drugs. The challenge now is to assess the clinical significance of the presence of deficient cells in tumors and to discover drugs that retain activity against MMR-deficient cells.

Introduction

Maintenance of genomic stability requires the proper functioning of DNA replication, repair, and recombination processes. Among these, MMR[3] plays a prominent role in the correction of replicative mismatches that escape DNA polymerase proofreading. MMR was originally described in bacteria that are able to repair mispair-containing bacteriophages (1). In the bacterial system, the important components are MutS, MutL, and MutH, named after their corresponding bacterial mutator strains. The MutS protein initially recognizes and binds to mismatched DNA (2). After this, MutH and MutL form a complex with MutS to carry out excision repair; MutH has endonuclease activity, but the specific activity of MutL has not been identified. The important role played by the MMR proteins is emphasized by the fact that they are highly conserved from bacteria to yeast to mammals. Biochemical and genetic studies in human cells have defined five genes whose products play key roles in MMR including *hMSH2* (3, 4), *hMSH3* (5, 6), and *hMSH6* [also called *GTBP* (7) or *p160* (8)], which are homologues of *MutS*, and *hMLH1* (9, 10) and *hPMS2* (11), which are homologues of *MutL* (Table 1). A sixth human gene, *hPMS1*, has also been suggested to be important for MMR, although biochemical studies supporting its involvement are not yet available (12). Both the bacterial and the eukaryotic systems direct repair to the newly replicated DNA strand, require multiple components, and can cut the strand to be repaired either upstream or downstream of the mismatch. hMSH2, either by itself (13) or when dimerized with either hMSH6 (7) or hMSH3 (14), binds small DNA mismatches (Fig. 1). The MutL homologues hMLH1 and hPMS2 form a heterodimer (15) and join the complex after the initial binding by either hMSH2-hMSH6 or hMSH2-hMSH3. Analysis of the mismatched nucleotide-binding specificity of the hMSH2-hMSH3 and hMSH2-hMSH6 protein complexes showed that they have overlapping but not identical binding specificity (14).

Loss of MMR causes destabilization of the genome and results in high mutation rates, particularly in microsatellite sequences in both noncoding (16, 17) and coding portions of the genome. Such sequences are found in the coding regions of the *HPRT* (18), *APRT* (19), *APC* (20), type II *TGF*-β (21), and *BAX* (22) genes, and mutation rates are increased at these loci in deficient cells. The majority of hereditary nonpolyposis colon cancer cases are due to underlying defects in either *MLH1* or *MSH2* (9, 10, 23, 24). Defects in *PMS1* or *PMS2* are less frequent (11, 24). Although the MMR system seems to be normal in the heterozygote cells containing a single functional gene copy, during carcinogenesis, the remaining wild-type allele is somatically mutated, resulting in the complete loss of MMR function in the tumor (4). In addition to hereditary nonpolyposis colon cancer, loss of MMR occurs frequently in many types of sporadic cancers as well, including endometrial, small and non-small cell lung, pancreatic, gastric, ovarian, cervix, and breast cancer (25–29). Mice that are deficient in either MLH1, MSH2, or PMS2 have microsatellite instability in many tissues and a predisposition to form tumors, especially lymphoma (30–33).

Recent studies have documented that loss of MMR is an important mechanism of resistance to a variety of clinically important drugs (Table 2), due in part to the fact that the MMR system can recognize and bind to various types of adducts in DNA as well as to mismatches. Rather than being a major effector of the removal of such adducts, the main role of the MMR seems to be as a detector of specific types of DNA damage. A schematic diagram of the proposed mechanism is presented in Fig. 1. This review will focus on how loss of MMR results in drug resistance.

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[3] The abbreviations used are: MMR, DNA mismatch repair; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; AGT, O^6-alkylguanine-DNA alkyltransferase.

Table 1 MutS and MutL homologues

Bacterial MMR	Human homologue	Chromosomal localization	Size (kDa)	Reference no.
MutS	hMSH2	2p22-21	105	3, 4
	hMSH3	5q11-13	127	5, 6
	hMSH6	2p16-15	153	7, 8
MutL	hMLH1	3p23-21	85	9,10
	hPMS1	2q31-33	106	11
	hPMS2	7p22	96	11

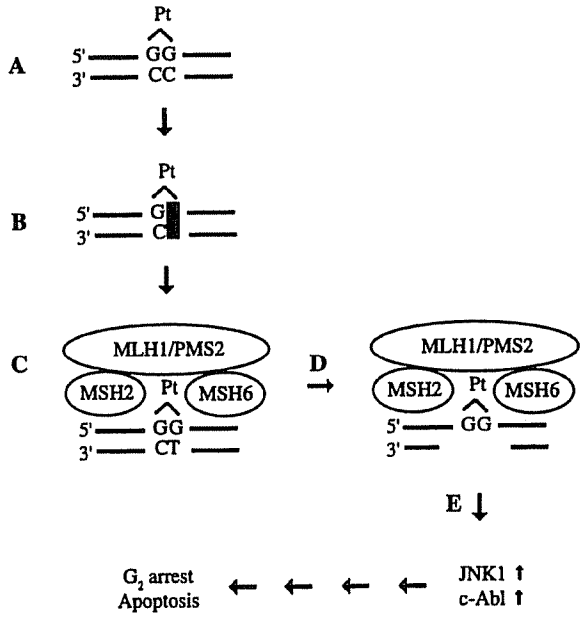

Fig. 1 Basic sequence of events that seem to be the basis for cytotoxicity of carboplatin and cisplatin: *A*, formation of the adduct; *B*, misincorporation of a base opposite the adduct at the next round of replication; *C*, recognition of the adduct/mispair by the MMR system; *D*, attempted futile repair; *E*, generation of a signal that triggers apoptosis. *Pt*, an intrastrand 1,2 d(GpG) adduct.

Alkylating Agents

There exist a number of cell lines with known inactivating mutations in the MMR genes. The human colorectal adenocarcinoma cell line HCT116, which is hMLH1 deficient due to a hemizygous mutation in *hMLH1* resulting in a truncated nonfunctional protein, exhibits microsatellite instability and does not correct mismatches in cell-free extracts (34, 35). Transfer of chromosome 3, on which the *hMLH1* gene is located, into HCT116 cells corrects the MMR defect, reverses the mutator phenotype, and sensitizes the cells to the methylating agent MNNG, indicating that restoration of functional MMR abolishes methylation tolerance (36). Similarly, the human endometrial adenocarcinoma cell line HEC59, which is hMSH2 deficient due to different mutations in each of the two *hMSH2* alleles (35), is resistant to MNNG when compared to a subline into which chromosome 2 containing a wild-type copy of *hMSH2* has been transferred (37). Furthermore, the human endometrial adenocarcinoma cell line HEC-1-A, which carries mutations in *hPMS2*, exhibits microsatellite instability and resistance to MNNG when compared to the MMR-proficient KLE cells (38).

Methylating agents such as MNU, MNNG, procarbazine, and temozolomide (an activated form of procarbazine) form a variety of adducts in DNA, among which O^6-methylguanine is the most cytotoxic. Although the MMR system does not seem to be able to recognize the alkylated guanine directly (39), it does recognize the O^6-methylguanine-thymine mispair that occurs after erroneous incorporation of a thymine rather than a cytosine opposite the O^6-methylguanine during the next cycle of DNA replication (40). One hypothesis is that having recognized the mismatch, the MMR system incises the thymine-containing strand; excises the thymine and surrounding bases, creating a gap; and then fills in the gap via repair synthesis. However, because a thymine is again incorporated opposite the persisting O^6-methylguanine, the site is once again recognized by the MMR system, and a new round of attempted repair is triggered. This futile cycling is envisioned as increasing the risk of a double-strand break at the time of the next S phase that could then trigger apoptosis (41). This model predicts that loss of MMR confers tolerance to methylating agents such as MNU and MNNG by virtue of the fact that the cell does not attempt repair. In addition to the experiments in which MMR was restored by chromosome transfer, this hypothesis is supported by the fact that selection of cells for resistance to MNU or MNNG frequently results in loss of MMR (42) and loss of guanine-thymine mismatch binding activity (40, 43).

Loss of MMR seems to be important as a mechanism of resistance to two drugs currently used in the clinic, both of which produce large numbers of O^6-methylguanine adducts, namely temozolomide, a monofunctional methylating imidazotetrazinone, and procarbazine, a methylhydrazine derivative (44–46). There is an interesting interplay between AGT and the MMR system that controls sensitivity to these agents. AGT is capable of transferring the alkyl group from the O^6 position of guanine in DNA to its active site cysteine residue, and in cells that express this enzyme, this is the major route by which these types of adducts are removed from the DNA. However, these alkylated guanines are only toxic to the cell if they are detected by the MMR system. For cells with normal MMR, high levels of AGT prevent the cytotoxic effect by removing the O^6-alkylguanine DNA adducts, and inactivation of AGT by a potent inhibitor, O^6-benzylguanine (47), sensitizes cells to killing by temozolomide. In MMR-deficient cell lines, inactivation of AGT fails to sensitize cells to temozolomide, suggesting that even large numbers of DNA adducts are not cytotoxic in the absence of the MMR detector, although they may be very mutagenic. Thus, MMR mutations seem to override the AGT mechanism of resistance (44); if the cell cannot detect the presence of the adducts in DNA, then it matters less how many such adducts are present. Human glioblastoma multiforme xenografts treated repeatedly with procarbazine during growth in nude mice developed drug resistance concurrently with a deficiency in MMR associated with loss of detectable hMSH2 expression (46). Although MMR-deficient cells are resistant to MNU, MNNG, temozolomide, and procarbazine, it is not yet known whether they are resistant to other chemotherapeutic methylating agents.

Table 2 The effect of loss of MMR on sensitivity to cytotoxic agents

Resistance (Ref. no.)	No effect (Ref. no.)	Hypersensitivity
Busulfan (46)	BCNU[a] (44, 48)	Unknown
Carboplatin (62)	Melphalan (48, 49)	
Cisplatin (59–62)	Oxaliplatin (62)	
Doxorubicin (61)	Paclitaxel (49)	
Etoposide (49)	Perfosfamide (49)	
Mercaptopurine (54)	Transplatin (62, 65)	
MNNG (36, 42)		
MNU (46)		
Procarbazine (46)		
Temozolomide (44–46)		
6-Thioguanine (53, 54)		

[a] BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

Dacarbazine, for example, produces N^7-methylguanine and N^3-methyladenine adducts that are repaired by the base excision repair system as well as O^6-methylguanine adducts.

Very recently a MMR-proficient human glioblastoma multiforme xenograft was reported to be responsive to busulfan when treated with an LD_{10} dose, whereas a procarbazine-selected MMR-deficient subline was not (46). This suggests that the adducts formed by busulfan might also be recognized as mismatches by the MMR proteins, either directly or after a round of replication and misincorporation of a base opposite the adduct. The adducts produced by busulfan are less well characterized than those for the other alkylating agents because of its lower reactivity and the relatively rapid depurination of the adducted bases. Additional studies are needed to document that loss of MMR is a cause of busulfan resistance.

Sensitivity to 1,3-bis(2-chloroethyl)-1-nitrosourea, a bifunctional chloroethylnitrosourea that forms O^6-chloroethylguanine DNA adducts, seems not to be affected by the MMR status of the cell (44, 48). Similarly, the nitrogen mustards melphalan and perfosfamide (an active form of cyclophosphamide) are not differentially cytotoxic to MMR-proficient and -deficient tumor cell lines (48, 49), suggesting that the DNA adducts produced by these agents are not recognized by the MMR protein complex.

6-Thioguanine and Mercaptopurine

The cytotoxicity of 6-thioguanine and mercaptopurine results primarily from their conversion to 2′-deoxy-6-thioguanosine triphosphate and subsequent incorporation into DNA (50). They characteristically produce delayed cytotoxicity (51) and a variety of chromosomal abnormalities including an increased rate of sister chromatid exchange (52). MMR-deficient cells are 5- to 10-fold more resistant to 6-thioguanine (49, 53). After incorporation into DNA, 6-thioguanine can be chemically methylated by *S*-adenosylmethionine to form S^6-methylthioguanine. This adduct is not a good substrate for AGT and therefore would be expected to persist in the DNA (54). Particularly when preceded by a 5′ cytosine, S^6-methylthioguanine paired with cytosine is recognized by hMutSα (40, 55). During DNA replication, S^6-methylthioguanine can pair with thymine as well as its normal partner, cytosine, and the resultant S^6-methylthioguanine-thymine pairs are also identified by the MMR system as replication errors (54). Studies of the replication of DNA containing 6-thioguanine suggest that it does not miscode with sufficient frequency for the toxicity to be ascribed to thioguanine-thymine bp alone (56), consistent with the hypothesis that attempted repair of either S^6-methylthioguanine-cytosine or S^6-methylthioguanine-thymine can result in cytotoxicity. The currently available data support the argument that it is the ability of the MMR system to recognize the abnormalities in the DNA produced by the incorporation of 6-thioguanine and attempt repair that triggers cytotoxicity. If the detector function of the MMR system is disabled, despite the fact that the adducts may persist in the DNA, cytotoxicity is markedly reduced.

Platinum Compounds

DNA is the primary intracellular target of cisplatin, and eukaryotic cells respond to the presence of cisplatin adducts in DNA by activating signal transduction pathways that result in cell cycle arrest and apoptosis (57). The first indication that MMR might be a determinant of sensitivity to cisplatin was the observation that introduction of *mutS* or *mutL* mutations into *Escherichia coli* already hypersensitive to cisplatin due to the presence of a *dam* mutation caused them to become resistant (58). Subsequently, it was demonstrated that in two unrelated cell systems, loss of either hMLH1 (HCT116 cells) or hMSH2 (HEC59 cells) function resulted in low-level cisplatin (59–61) and carboplatin resistance (62), and that some human tumor cell lines selected for resistance to cisplatin [*e.g.*, 2008/A (63)] exhibit microsatellite instability (59, 60) and are defective in strand-specific MMR (61). The fact that loss of MMR results in resistance to both cisplatin and carboplatin was not unexpected, because although carboplatin contains a 1,1-cyclobutanedicarboxylato-leaving group and undergoes aquation more slowly, the structures of the aquated forms of cisplatin and carboplatin are the same, as are the types of adducts.

Pure hMSH2 has been reported to bind to platinated DNA in mobility shift assays (64). Human MutSα, a heterodimer of hMSH2 and hMSH6, has been shown to bind to DNA containing adducts produced by cisplatin (65) and has greatest affinity for lesions in which a thymine has been misincorporated opposite the 3′ guanine (66). Adducts formed by oxaliplatin or transplatin are not recognized by the MMR system (62, 65). Oxaliplatin and transplatin do not differ in their cytotoxicity to MMR-proficient and -deficient tumor cell lines (62), suggesting that the components of the MMR system responsible for the difference in sensitivity are quite specific in their ability to discriminate between different types of closely related DNA adducts.

It is not yet clear how the platinum adducts are recognized by the MMR proteins. It has been demonstrated biochemically that hMSH2 can bind to a 1,2 d(GpG) adduct, but it is also possible that the MMR system recognizes monoadducts, monoadducts modified by reaction with glutathione, or interstrand adducts as well. It is possible that cisplatin adducts distort the DNA in a manner that mimics the presence of either a single-base mismatch or an insertion/deletion mispair. The current paradigm is that, as for the methylating agents, the MMR system serves as a detector for cisplatin-damaged DNA; resistance is thought to result from failure of the cell to recognize the adducts and to activate signaling pathways that trigger apoptosis (Fig.

1). If this paradigm is correct, then the detector must be able to initiate activation of signaling pathways. Indeed, it has recently been reported that cisplatin activates c-*jun* NH_2-terminal kinase 1 by a p21-activated kinase protein 65 and mitogen-activated protein kinase kinase 4-independent mechanism more efficiently in MMR-proficient cells than in MMR-deficient cells, and that cisplatin activates c-Abl kinase in the MMR-proficient cells, whereas this response is completely absent in MMR-deficient cells (67). This reveals that activation of c-*jun* NH_2-terminal kinase 1 and c-Abl by cisplatin is in part dependent on the integrity of the MMR function, suggesting that these kinases are part of the signal transduction pathway activated when MMR proteins recognize cisplatin adducts in DNA.

Other Chemotherapeutic Agents

Loss of MMR has been shown to be associated with low-level resistance to etoposide (49) and doxorubicin (61). How loss of MMR produces low-level resistance to these topoisomerase II inhibitors is less clear than for those agents that react directly with DNA to produce adducts that distort its structure in a manner similar to that of true DNA mismatches. It may be that the MMR proteins serve as a detector of the cleavable complex (68) produced by the binding of etoposide or doxorubicin to topoisomerase II or that the MMR proteins normally act to stabilize the drug-induced cleavable complex on the DNA and thus serve to augment the DNA damage. Additional studies are required to document the interactions between the MMR proteins and topoisomerase II. The observation that there is no difference in the cytotoxicity of paclitaxel between MMR-proficient and -deficient cells (49) is consistent with the fact that this agent is not known to interact with DNA at all.

MMR and the G_2-M-Phase Cell Cycle Checkpoint

It has been suggested that the MMR system is involved in promoting G_2 cell cycle arrest and cell death after treatment with MNNG in cells that are MMR proficient (69). Similarly, a G_2 arrest has been reported in MMR-proficient cells after treatment with 6-thioguanine (53). In contrast, the same exposure to 6-thioguanine did not induce a G_2 arrest but rather just a G_1 delay in MMR-deficient cells. The arrest at the G_2 cell cycle checkpoint may permit the cell to attempt repair of DNA mismatches and prevent the replication of mutated DNA, similar to the arrest at the G_1-S-phase checkpoint mediated by p53 and p21 in response to DNA damage. The signal generated by the detection of MNNG or 6-thioguanine damage by the MMR system is unknown, although there is some evidence that it works via regulation of the $p34^{cdc2}$-cyclin B complex (70). Very recently, it has been reported that MLH1-deficient human tumor cell lines also fail to engage G_2 cell cycle arrest after cisplatin damage (71). The link between the MMR system and G_2 arrest suggests that the MMR system is involved not only in the repair of true mismatches but also in processes that limit the replication of cells when DNA damage is detected.

Clinical Significance

Loss of MMR can result in drug resistance directly by impairing the ability of the cells to detect damage and indirectly by increasing the mutation rate at loci that mediate resistance to other classes of drugs. Although, by the direct route, loss of MMR results only in relatively small degrees of resistance to the platinum-containing drugs, several lines of evidence suggest that this resistance is nevertheless of substantial biological and clinical significance: (*a*) this low-level resistance to cisplatin has been reported to be sufficient to produce progressive enrichment for MMR-deficient tumor cells during treatment *in vitro* (72); (*b*) $MSH2^{+/+}$ embryonic stem cells (31) grown as xenografts have been shown to be responsive to treatment with a single LD_{10} dose of cisplatin, whereas isogenic $MSH2^{-/-}$ tumors were not, suggesting that the degree of cisplatin resistance conferred by loss of MMR is sufficient to produce a large difference in clinical responsiveness *in vivo* (72). Similarly, a MMR-proficient glioblastoma multiforme xenograft has been shown to be much more responsive to treatment with procarbazine, temozolomide, and busulfan than a procarbazine-selected MMR-deficient subline (46); (*c*) loss of MMR has been reported in tumor cell lines selected for resistance to cisplatin (60) or doxorubicin (61); and (*d*) the frequency of positive immunoblot analysis for hMLH1 protein in ovarian carcinomas obtained after chemotherapy with a cisplatin or carboplatin-containing regimen was shown to be substantially lower than the frequency among tumors sampled before treatment (71). Although the samples were not paired, and therefore two different groups of patients were compared, this loss of hMLH1 expression is consistent with the concept that treatment with platinum drugs can enrich for MMR-deficient cells.

The issue of when loss of MMR occurs during oncogenesis remains controversial even for hereditary nonpolyposis colon cancer, which represents the best-defined clinical situation (73). However, once such cells are present in the tumor, their genomic instability may result in the accumulation of additional mutations that contribute to the phenomenon of tumor progression. Enrichment of these cells as a result of chemotherapy would be expected to accelerate this process. Indeed, microsatellite instability, a hallmark of the genomic instability due to loss of MMR (74), has been reported to be present in up to 94% of the patients with therapy-related leukemia or myelodysplastic syndromes, consistent with drug-induced enrichment for genetically unstable cells (75).

Overview

A coherent picture of how MMR mediates cytotoxicity has emerged from studies of the mechanism of action of the methylating agents, 6-thioguanine, and cisplatin in particular. In each case, the MMR system recognizes the damaged bases or the mismatch that results from attempted replication across the damaged base. Recognition is followed by events, not yet well defined, that generate a signal capable of activating apoptosis. Operationally, the MMR system is thus functioning as a detector; when the detector is disabled, the cell cannot sense the damage present in its DNA, the apoptotic cascade is not activated, and the cell is phenotypically drug-resistant. Exactly how the MMR system generates a proapoptotic signal is not yet clear. The DNA lesions produced by methylating agents, 6-thioguanine, and cisplatin are all quite mutagenic, so that it is reasonable to expect that cells that fail to die because the MMR detector is disabled will have very high mutation rates not only

because of the failure to repair DNA polymerase errors, but also because of the persistence of the adducts in the DNA. Clinical studies of the significance of MMR-deficient cells in tumors with respect to the rate of development of drug resistance are now urgently needed.

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EXHIBIT B

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DNA Damage Signals Induction of Fas Ligand in Tumor Cells

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ABSTRACT

Many anticancer agents exert their cytotoxicity through DNA damage and induction of apoptosis. Fas ligand (FasL), a key component of T lymphocytes, has been shown to be induced by some of those agents. To address what is an early signal for this induction, we constructed a FasL promoter-luciferase reporter gene to investigate effects of DNA topoisomerase (Topo) II inhibitors on FasL promoter activity. Transient transfection assays in HeLa and other tumor cell lines demonstrated that induction of FasL promoter activity in response to Topo II inhibitors such as VM-26 mimicked endogenous FasL expression under the same conditions. The ability of these agents to induce FasL expression correlated with their ability to cause DNA damage. For instance, complex-stabilizing Topo II inhibitors such as etoposide, teniposide, and doxorubicin, which cause DNA damage, strongly induce FasL expression; by contrast, non-DNA-damaging catalytic Topo II inhibitors such as ICRF-187 and merbarone do not do this. In support of the notion that DNA damage triggers FasL induction, we found that DNA-damaging irradiation also induced FasL promoter activity in a dose-dependent manner. Finally, the catalytic Topo II inhibitor ICRF-187 suppressed VM-26-induced-FasL expression. This suppression correlated with the ability of this drug to inhibit VM-26-induced DNA strand breaks. Together, our results suggest that DNA damage in response to agents such as etoposide and teniposide might serve as an early signal to induce FasL expression.

DNA topoisomerases (Topos) are nuclear enzymes that regulate DNA topology and are required for DNA replication and transcription (Nelson et al., 1986; Brill et al., 1987). These enzymes are also implicated in chromosome segregation, DNA repair, cell cycle progression, and RNA processing (Rose and Holm, 1993; Holloway, 1995; Sekiguchi and Shuman, 1997). Eukaryotic cells express two forms of topoisomerases (D'Arpa et al., 1988; Tsai-Pflugfelder et al., 1988). The type I form (Topo I) is an ATP-independent enzyme that catalyzes DNA relaxation via transient single-stranded DNA breaks (D'Arpa et al., 1988). By contrast, the type II form (Topo II) is an ATP-dependent enzyme that catalyzes knotting-unknotting and catenation-decatenation reactions by the breakage, strand-passage, and reunion of double-stranded DNA (Tsai-Pflugfelder et al., 1988). Because of their essential role in DNA replication and cell growth, as well as their high level of expression in proliferating cells, these enzymes are ideal targets for cancer chemotherapy (Heck and Earnshaw, 1986; Liu, 1989). Topo II inhibitors are among the most useful anticancer drugs for many types of cancer (Liu, 1989; Osheroff et al., 1994).

Mechanistically, the catalytic cycle of Topo II features a minimum of four distinct steps: 1) DNA binding by the enzyme, 2) DNA cleavage, 3) strand passage, and 4) religation and enzyme turnover (Osheroff et al., 1994). Several well-characterized Topo II inhibitors include doxorubicin, teniposide (VM-26), and etoposide (VP-16). These drugs appear to bind to the Topo II-DNA complex and inhibit the religation of the broken DNA strands, thus inducing protein-associated DNA strand breaks through stabilization of the covalently linked Topo II/DNA-cleavable complexes. Hence, they have been known as cleavable complex-stabilizing inhibitors or Topo II poisons. A consequence of these drug actions is interference with transcription, DNA synthesis, and mitosis, eventually leading to cell death by apoptosis (Fisher, 1994). By contrast, other Topo II inhibitors, such as the bisdioxopiperazine derivatives and merbarone, do not stabilize DNA-enzyme cleavable complexes, although they also target the enzyme and inhibit its activity; these are catalytic inhibitors of the enzyme. For instance, dioxopiperazine derivatives are believed to bind to Topo II at a stage when religated double-stranded DNA is still locked in the enzyme, thereby inhibiting the enzymatic activity because the bound enzyme cannot initiate a new round of catalysis (Sehested and Jensen, 1996). Based on their action on the enzyme, the catalytic Topo II inhibitors are generally considered to be non-DNA-damaging agents. In addition, compared with the complex-stabilizing inhibitors of Topo II, the catalytic Topo II

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ABBREVIATIONS: Topo, DNA topoisomerase; FasL, Fas ligand; PARP, poly(ADP-ribose)polymerase; Z-VAD.fmk, Z-Val-Ala-DL-Asp-fluoromethylketone; CHO, Chinese hamster ovary.

inhibitors have been shown to antagonize the actions of the cleavable complex-stabilizing Topo II inhibitors (Sehested et al., 1993). Thus, pretreatment with ICRF-187, a dioxopiperazine derivative, reduced VP-16- or daunorubicin-induced DNA breaks and apoptosis (Sehested et al., 1993), presumably because the former competes with VP-16 and daunorubicin for the enzyme, making the enzyme less available for VP-16 and daunorubicin and, thus, decreasing DNA damage.

It has been shown recently that some of the Topo II inhibitors induce Fas ligand (FasL) expression (Friesen *at el.*, 1996). FasL is a 37-kDa protein that belongs to the type II protein superfamily and is expressed predominantly in activated T cells. The Fas/FasL system plays a pivotal role in the regulation of a variety of immunological processes by activating the apoptotic pathway (Ju et al., 1995; Nagata and Golstein, 1995). Binding of Fas to FasL triggers activation of a series of proteases, including interleukin-converting enzymes, and DNA fragmentation, leading to cell death by apoptosis (Lowin et al., 1994). This type of cell death induced by Fas/FasL interactions has been implicated in the elimination of excess activated T cells in the peripheral circulation and in the killing of tumor targets by immune cytotoxic effector cells (Ju et al., 1995).

Although much of attention has been directed to FasL expression in response to T cell activation (Rouvier et al., 1993; Herr et al., 1997), less is known about its regulation by anticancer agents, particularly at the transcriptional level. In this study, we asked what is an early signal for FasL induction in response to Topo II inhibitors.

Materials and Methods

Cell Culture and Transfection. All cell culture media (BioWhittaker, Walkersville, MD) were supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 U of penicillin/ml, and 100 mg of streptomycin/ml (Life Technologies, Gaithersburg, MD). Human leukemic CCRF-CEM (CEM) cells (Kusumoto et al., 1996) were grown in supplemented (Eagle's) minimum essential medium. Jurkat and Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640; HeLa cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium. Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO_2.

The FasL promoter-luciferase reporter plasmid was introduced into CEM cells by electroporation using the Gene Pulser II apparatus with an extender (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Briefly, about 5 million exponentially growing cells were harvested, washed twice with phosphate-buffered saline, and resuspended in 400 μl of supplemented (Eagle's) minimum essential medium without fetal bovine serum. Twenty micrograms of plasmid was used per electroporation. The same amount of pSV-β-galactosidase reporter (Promega, Madison, WI) was cointroduced into the cells in each electroporation experiment to normalize for transfection efficiency. After electroporation, cell suspensions were placed at room temperature for 10 min and then transferred to a T-25 flask with 12 ml of culture medium and incubated overnight at 37°C before drug treatment.

For transfection of adherent cells (HeLa and CHO), plasmid DNA was introduced into cells by the calcium phosphate method (Jordan et al., 1996). Briefly, 20 μg each of FasL promoter-luciferase construct and pSV-β-galactosidase were used per 100-mm dish containing ~50% confluent cells. After the addition of the transfection solution containing the plasmid DNA to medium, cells were incubated overnight at 37°C. Cells were then subcultured in 12-well plates with 2 ml of medium per well and grown at 37°C overnight before treatment with drugs or radiation.

Chemicals. VM-26, VP-16, doxorubicin, ICRF-187, and merbarone have been described previously (Kusumoto et al., 1996). Bisbenzimide (Hoechst 33342) was purchased from Sigma Chemical. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD.fmk) was purchased from Bachem (Torrance, CA). [^{14}C]Thymidine was purchased from Amersham (Arlington Heights, IL).

Polymerase Chain Reaction Cloning. Genomic DNA was isolated from CEM cells by a standard method (Ausubel et al., 1989). Polymerase chain reactions (PCRs) for amplification of the FasL promoter region were performed according to a standard method (Ausubel et al., 1989) using a commercial kit (AmpliTaq; Perkin-Elmer, Foster City, CA). Primers for PCR were FasLp5.7, 5′-CTCGAGTTCTGATATTTCAAAACAGAATAG (sense) (Holtz-Heppelmann et al., 1998), containing the restriction enzyme *Xho*I site, and FasLp3.1, 5′-AAGCTTATGGCAGCTGGTGAGTCAGG (antisense), containing a *Hin*dIII site for unidirectional cloning into pGL2-Basic (Promega). PCR products were first cloned into PCR2.1 (InVitrogen, Carlsbad, CA) and then subcloned into pGL2-Basic. To verify the cloned DNA fragment, nucleotide sequences were determined by Sequenase kit version 2 (Amersham).

Immunoblot Analysis. Cellular proteins were extracted with lysis buffer (Keane et al., 1996) from exponentially growing cells. Protein concentration was determined using the Bio-Rad protein assay kit. Protein samples were separated in 9% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane using a semidry transfer apparatus (Hoefer Scientific, San Francisco, CA). The membrane was blocked in 5% dry milk in TBS (50 mM Tris, pH 7.4, 0.87% NaCl) and then incubated with specific antibodies for 1 h at room temperature or overnight at 4°C with gentle shaking. After three washes with TBS, secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) were added to the membranes and incubated for 1 h under the same conditions. After a final three washes with TBS, immunoblots were developed with an enhanced chemiluminescence (ECL) detection method (Amersham). To normalize for protein loading and transfer, anti-β-tubulin antibody (Oncogene Research, Cambridge, MA) was used on the same membrane. Antibodies were purchased from Upstate Biotechnology (polyclonal anti-PARP; Lake Placid, NY), Transduction Laboratory (monoclonal anti-FasL antibody; Lexington, KY), or Santa Cruz Biotechnology (polyclonal anti-FasL antibodies; Santa Cruz, CA).

Drug Treatment and Irradiation. After transfection, adherent cells were trypsinized, subcultured into 12-well plates with 2 ml of medium per well, and grown overnight. Drugs were then added to the medium at concentrations as indicated in *Results*. Cells were harvested for luciferase assays 24 h after drug treatment. For UV irradiation, medium was removed immediately before treatment; cells were then exposed to UV at a defined energy level using Stratalinker (Stratagene, La Jolla, CA), and fresh medium was added back after the UV treatment. Cells were then grown for another 24 h, harvested for luciferase assays, and lysed in 100 μl of 1× luciferase assay buffer (Promega). Suspension cells, after electroporation, were grown in 12 ml of medium in T-25 flasks overnight and then divided into 12-well plates with 2 ml of culture medium per well and treated with drug as above. Luciferase activity was assayed in a luminometer (Turner Designs, Sunnyvale, CA), and normalized by β-galactosidase activity for each treatment.

Detection of Apoptosis and Cytotoxicity Assays. Apoptosis was determined by nuclear staining with Hoechst dye. HeLa cells were treated with drugs or UV irradiation for 24 h, trypsinized as usual, and incubated in a fixing solution (methanol/acetic acid, 3:1) for 15 min at room temperature before transferring to a glass slide. After briefly drying the slides, cells were stained with Hoechst dye (1 μg/ml) and examined under a fluorescent microscope (Zeiss, Thorn-

wood, NY). Any cells displaying shrunken nuclear structures with intense staining were scored as apoptotic cells, and the percentage of apoptosis was determined from a total of 200 cells per treatment. Cytotoxicity assays were done using trypan blue exclusion as suggested by the manufacturer (Life Technologies, Gaithersburg, MD).

Alkaline Elution. Alkaline elution assays for single-stranded DNA breaks were carried out essentially as described by Beere et al. (1996). Briefly, HeLa cells were labeled with 0.1 μCi of [^{14}C]thymidine/ml for 24 h and then treated with drugs for 1 h before harvesting of them for alkaline elution assays. To test the effect of ICRF-187 on VM-26-induced DNA strand breaks, labeled HeLa cells were pretreated with 100 μM ICRF-187 for 1 h, followed by an additional 1-h treatment with VM-26 (10 μM) before harvesting for alkaline elution assays.

Results

Up-Regulation of FasL Protein and FasL Promoter Activity in Different Types of Tumor Cells Treated with Topo II Inhibitor VM-26. It was reported previously that anticancer agents can induce FasL expression (Friesen *at el.*, 1996). Consistent with these results, we observed up-regulation of FasL in VM-26- or doxorubicin-treated CEM and Jurkat cells by Western blot (Fig.1A). To further investigate FasL induction in response to anticancer drugs, we cloned the FasL promoter (~1200 bp) from CEM cells and verified by DNA sequencing that it was identical with the published sequence (Holtz-Heppelmann et al., 1998). A FasL promoter-luciferase reporter construct was then made in pGL2-Basic. Different drug concentrations were chosen in this experiment for different cell lines because initial dose variation experiments showed that at these concentrations, the highest level of FasL promoter activity was achieved for a particular cell line. After introduction of this construct into CEM cells by electroporation, followed by VM-26 treatment at 1 μM, we found a moderate (~2-fold) induction of FasL promoter activity (Fig. 1B). To test whether this is a cell-specific phenomenon, we introduced the same construct into HeLa and CHO cells, respectively; treatment of these cells with VM-26 at 10 μM resulted in FasL induction in both cell lines. Interestingly, the induction level was much greater than that in CEM cells, apparently due in part to a higher transfection efficiency for both HeLa and CHO cells. As shown in Fig.1B, treatment of these cells for 24 h with 10 μM VM-26 yielded about a 12-fold increase in FasL promoter activity compared with the DMSO control; similarly, an approximate 6-fold induction was observed in CHO cells. No more than a 1.5-fold (CEM cells) or 2.3-fold (HeLa and CHO cells) increase in luciferase activity was detected for the empty vector pGL2-Basic under these conditions. Standard deviations are less than 1. Because HeLa cells seem to produce higher levels of FasL promoter activity than the other cell lines, we used this cell line for subsequent experiments.

To test the effect of drug concentration on endogenous FasL protein in HeLa cells, we treated HeLa cells for 24 h with VM-26 at 0, 0.1, 0.5, 1, 5, and 10 μM, respectively. As shown in Fig. 2A, HeLa cells expressed FasL protein in the absence of drug, but the amount of FasL protein increased with the concentration of VM-26. We also examined FasL

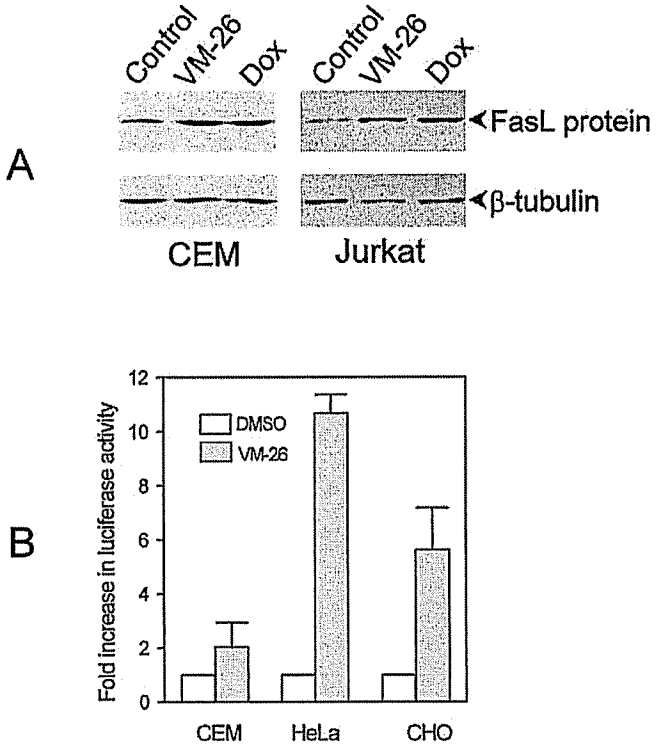

Fig. 1. Induction of FasL expression by Topo II inhibitors VM-26 and doxorubicin (Dox). A, representative Western blot showing expression of FasL in CEM and Jurkat cells treated with VM-26 (1 μM) or doxorubicin (0.2 μM) for 24 h. B, VM-26-induced FasL promoter activity in CEM (1 μM), HeLa (10 μM), and CHO (10 μM) cells. Cells were transfected with the FasL promoter-luciferase construct, treated with drugs, and harvested for luciferase assays as described in *Materials and Methods*. FasL promoter activity is expressed as luciferase activity relative to the control [DMSO (dimethyl sulfoxide)] activity set at one. Values are means ± S.D. of three independent experiments.

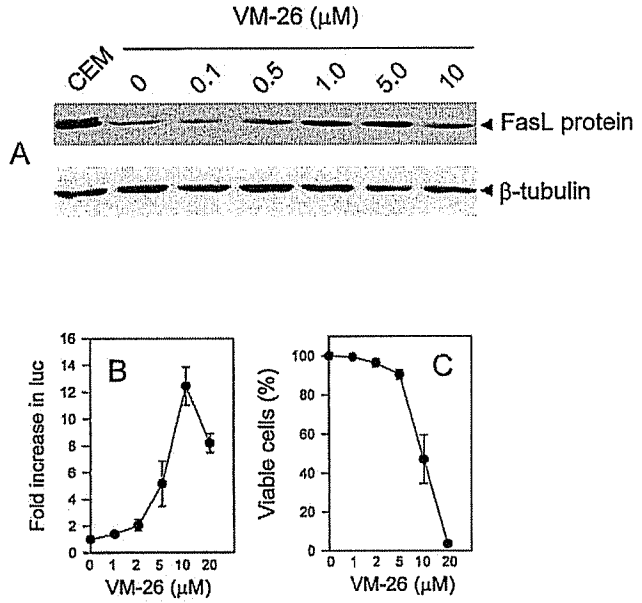

Fig. 2. Effect of VM-26 concentration on endogenous FasL protein (A), FasL promoter activity (B), and cell killing (C) in HeLa cells. A, expression of endogenous FasL protein was examined by Western blotting. Untransfected HeLa cells were treated with VM-26 as above and harvested for protein analysis after 24 h. CEM cells without drug treatment served as a positive control. B, FasL promoter activity in response to VM-26 at indicated concentrations. HeLa cells were transfected with the FasL promoter-luciferase construct and treated with VM-26 for 24 h before harvesting for luciferase assays. C, VM-26 induced cytotoxicity as determined by trypan blue staining. Data in B and C are means ± S.D. of three independent experiments

promoter activity under similar conditions (Fig. 2B). At 2 μM, the FasL promoter activity increased about 2-fold, and the maximal induction was observed at 10 μM and then seen to decrease at 20 μM; concentrations of VM-26 of 10 μM or higher led to substantial cell death (Fig. 2C).

Induction of FasL Promoter Activity by DNA-Damaging Agents. To test whether FasL induction has any specificity, we examined other Topo II inhibitors (doxorubicin, VP-16, merbarone, and ICRF-187). Both doxorubicin and VP-16, like VM-26, stabilize DNA-protein complexes and cause DNA damage and strand breaks (Liu, 1989). These drugs induced FasL promoter activity (Fig. 3, A and B), although the ability to do so varied among them. However, the catalytic Topo II inhibitors ICRF-187 and merbarone, which do not directly damage DNA (Sehested et al., 1993), caused no significant induction of FasL promoter activity (Fig. 3, D and E). ICRF-187 induced less than 2-fold increase in FasL promoter activity at up to 300 μM (Fig. 3E), although cytotoxicity to HeLa cells of ICRF-187 at 300 μM (Fig. 3F) is slightly higher than that of VM-26 at 5 μM (Fig 2C). Thus, it appears that induction of FasL is associated with DNA damage. To test this, we asked whether other DNA-damaging agents with different modes of action can induce FasL expression. As expected, UV irradiation induced FasL promoter activity. The minimal dose that caused FasL induction was 2 mJ/cm^2 when assays were carried out 24 h after UV treatment; peak activity was observed at 10 mJ/cm^2 (Fig. 3C). Like UV irradiation, γ irradiation also induced FasL promoter activity in a dose-dependent manner at the range of 0 to 10 Gy (data not shown).

FasL-Inducing Agents Cause Apoptosis, but FasL Induction Is Not a Consequence of Apoptosis. In addition to FasL induction, the DNA-damaging agents caused apoptosis, as indicated by cleavage of poly(ADP-ribose)polymerase (PARP), a commonly used indicator of apoptosis. At the concentration or energy level that resulted in highest level of FasL promoter activity, we observed significant amount of cleaved PARP (85 kDa) (Fig. 4). By contrast, no PARP cleavage was detected for HeLa cells treated with the catalytic Topo II inhibitors, ICRF-187 and merbarone. Consistent with these data, we also observed that about 50% of cells treated with 10 μM VM-26 for 24 h exhibited shrunken nuclei as revealed by Hoechst dye staining, a feature of apoptosis, whereas no significant apoptosis was seen for cells treated with either ICRF-187 or merbarone.

The results from Figs. 1 through 4 suggest that there is an association of apoptosis and induction of FasL promoter activity, but whether the FasL induction is due to apoptosis or other signals before apoptosis is not clear. Consequently, we followed the time courses of FasL induction related to apo-

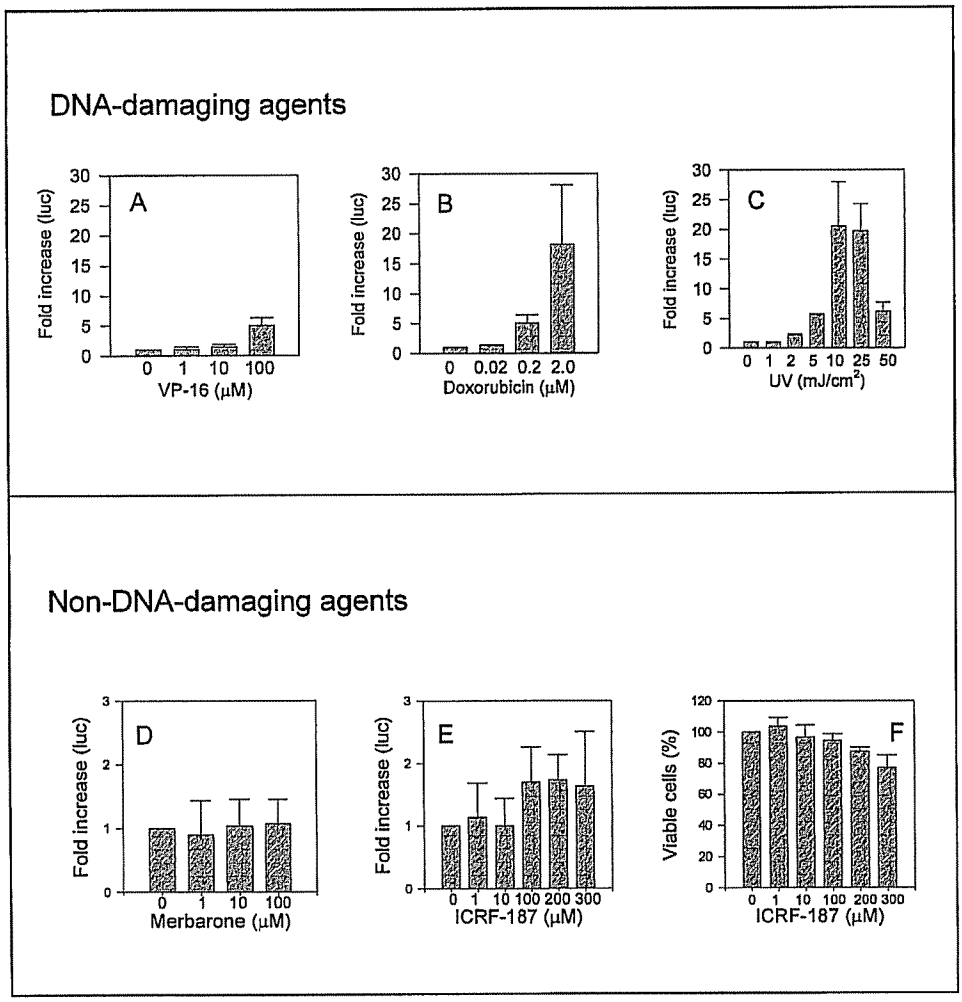

Fig. 3. Induction of FasL promoter activity by different classes of Topo II inhibitors and UV irradiation. HeLa cells were transfected with the FasL promoter-luciferase construct, treated with drugs or UV-irradiated at indicated doses, and harvested for luciferase assays as detailed in *Materials and Methods*. Cytotoxicity assays for ICRF-187 were done by trypan blue staining. Data are means ± S.D. of three separate experiments.

ptosis to determine the temporal order of these two events. We chose 10 μM for VM-26 and 10 mJ/cm^2 for UV irradiation because both conditions gave the highest level of FasL induction from previous experiments. We detected FasL induction in VM-26-treated cells at 12 h, when we did not detect significant apoptosis. Similarly, UV treatment also induced FasL expression before detection of apoptosis (Fig. 5). This suggests that FasL induction occurs earlier than apoptosis.

However, because there was a stage of overlap between apoptosis and FasL induction, we could not determine whether apoptosis was involved in the late stages of FasL induction. Therefore, we questioned whether any FasL induction could be detected when apoptosis is blocked. Z-VAD.fmk is a broad-spectrum apoptotic inhibitor used to block apoptosis mediated by a variety of agents. At either 50 or 100 μM, this inhibitor blocked apoptosis by VM-26 but had no effect on FasL induction (Fig. 6). These data suggest that FasL induction is an early event in response to drug treatment and is independent of apoptosis.

Catalytic Topo II Inhibitor ICRF-187 Suppresses VM-26-Induced DNA Strand Breaks and FasL Promoter Activity. What, then, triggers FasL induction as a result of drug treatment? Because our results demonstrated that only DNA-damaging agents induced FasL expression, we asked whether DNA damage caused by these agents signals FasL induction. To address this question, we took advantage of the fact that bisdioxopiperazine derivatives can suppress DNA-protein complex formation by cleavable complex-stabilizing agents such as VM-26, thus relieving DNA damage (Sehested et al., 1993; Jensen and Sehested, 1997). We pretreated HeLa cells with ICRF-187 for 1 h and then added VM-26 (10 μM). We found that ICRF-187 at 20 μM inhibited VM-26-stimulated FasL induction by 25% compared with the control (Fig. 7). When the concentration of ICRF-187 was increased to 100 μM, FasL promoter activity was decreased by 50% (Fig. 7). Even when cells were treated with both drugs at the same time, we still observed a significant inhibition of FasL induction, although at a lower level (about 10% inhibition at 100 μM ICRF-187). To confirm that this suppression was due to reduction of VM-26-induced DNA strand breaks by ICRF-187, we performed alkaline elution assays and found that pretreatment with ICRF-187 suppressed VM-26-induced DNA strand breaks, which was consistent with results of others (Sehested et al., 1993; Beere et al., 1996). Together, our data suggest that DNA damage caused by VM-26 might trigger the induction of FasL.

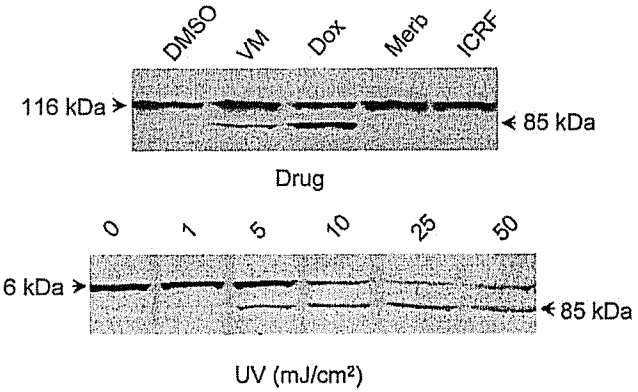

Fig. 4. Effect of Topo II inhibitors and UV irradiation on PARP cleavage. HeLa cells were treated with VM-26 (VM; 10 μM), doxorubicin (Dox; 2 μM), merbarone (Merb; 100 μM), or ICRF-187 (ICRF; 100 μM) for 24 h before harvesting for protein or UV irradiated at indicated energy levels as detailed in *Materials and Methods*, and then incubated for 24 h before protein extraction. Shown is a representative Western blot as revealed by anti-PARP antibodies. DMSO, dimethyl sulfoxide.

Discussion

FasL has been implicated in apoptosis and the cytotoxic effect of T lymphocytes. Expression of FasL, however, is not restricted to T lymphocytes. In particular, up-regulation of FasL has been found in some tumor cells (Hahne et al., 1996; Niehans et al., 1997), and we demonstrated in this study that

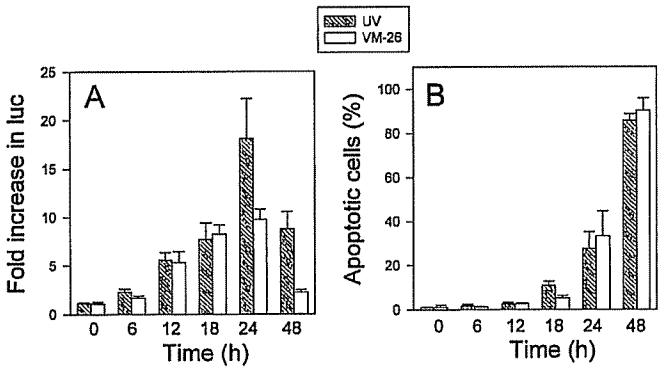

Fig. 5. Temporal induction of FasL promoter activity (A) and apoptosis (B) by VM-26 and UV irradiation. HeLa cells were transfected with the FasL promoter-luciferase construct, treated with 10 μM VM-26 or UV-irradiated (10 mJ/cm^2), and then harvested at indicated time points for luciferase assays or nuclear staining with Hoechst dye. All data are means ± S.D. of three independent experiments.

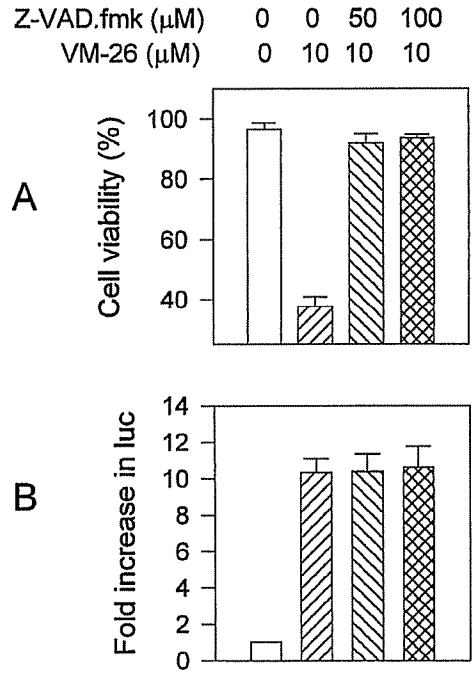

Fig. 6. Effect of Z-VAD.fmk on VM-26-induced cytotoxicity (A) and FasL promoter activity (B). HeLa cells were treated with Z-VAD.fmk at 50 or 100 μM for 1 h. VM-26 (10 μM) was then added to the cultures, and cells were incubated for another 24 h before harvesting for cytotoxicity assays (trypan blue exclusion) or luciferase assays. Z-VAD alone has no significant effect on either FasL promoter activity or cytotoxicity of HeLa cells. All data are means ± S.D. of three independent experiments.

nonhematopoietic tumor cells such as HeLa also express FasL. It is well known that a variety of stimuli induce expression of FasL. Because of the important role of FasL in apoptosis and regulation of immunological processes, its expression in response to these stimuli, particularly T cell receptor activation, has caught much attention (Latinis et al., 1997; Holtz-Heppelmann et al., 1998). On the other hand, induction of FasL by anticancer drugs has been reported recently, but the underlying mechanisms behind this phenomenon are far from clear. Accordingly, to better understand FasL gene regulation in response to anticancer agents, we asked here about the role of different types of clinically important anticancer drugs, Topo II inhibitors, in FasL expression by examining their effect on a FasL promoter-luciferase reporter. We found that induction of FasL promoter activity mimicked the expression of the endogenous FasL gene as result of Topo II inhibitor treatment (Figs. 1 and 2), supporting the notion that the FasL promoter reporter is a good indicator of FasL expression in response to these agents, as demonstrated in T cell receptor activation (Latinis et al., 1997) as well as drug induction (Kasibhatla et al., 1998).

Several anticancer drugs have been shown previously to induce FasL expression, and among them are Topo II inhibitors. To extend these observations, we tested several Topo II inhibitors representing different classes and mechanisms of inhibition of this enzyme. Our results indicated that FasL induction is drug specific. For instance, although cleavable complex-stabilizing Topo II inhibitors, such as VM-26, are strong inducers of FasL expression, catalytic Topo II inhibitors have little or no activity in this system. Interestingly, the level of FasL induction appeared to correlate with the ability of the drugs to induce DNA damage. Consistent with these results, we found that DNA-damaging agents, UV irradiation or γ-irradiation, also induced FasL expression, although these agents differ from the Topo II inhibitors in the way in which they cause DNA damage. Importantly, our results indicate that there is a relationship between DNA damage and FasL induction. In support of this notion, our results with ICRF-187, a catalytic Topo II inhibitor that has been shown in this study and by others (Sehested et al., 1993; Beere et al., 1996) to suppress complex inhibitor-induced DNA damage, demonstrated that this agent also inhibited VM-26-induced FasL expression. Together, these results suggest that DNA damage caused by these agents triggers FasL induction.

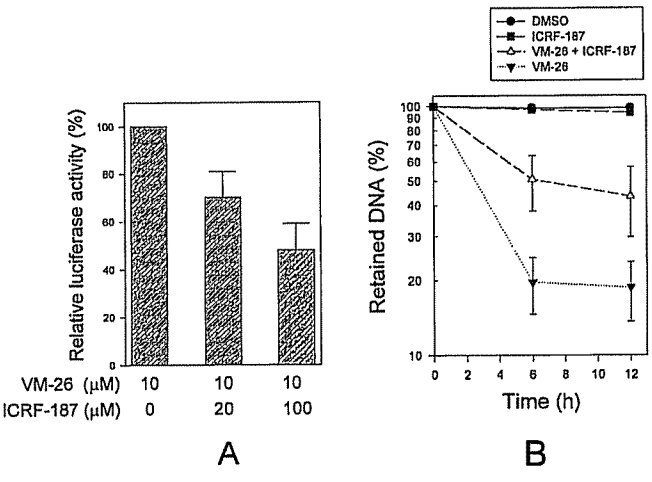

Fig. 7. Effect of ICRF-187 on VM-26-induced FasL promoter activity and DNA strand breaks. A, ICRF-187 suppresses VM-26-induced FasL promoter activity. HeLa cells were transfected with the FasL promoter-luciferase construct and pretreated with ICRF-187 at 20 or 100 μM for 1 h before addition of VM-26 (10 μM). Cells were incubated for another 24 h and then harvested for luciferase assays. B, alkaline elution assays for DNA strand breaks induced by VM-26. HeLa cells were labeled with [^{14}C]thymidine and analyzed for DNA damage. See *Materials and Methods* for details. All data are means ± S.D. of three independent experiments.

The mechanism or mechanisms by which ICRF-187 inhibits DNA damage by complex-stabilizing Topo II inhibitors are not fully understood, but it is believed to involve the different stages of the Topo II catalytic cycle at which these two classes of inhibitors target the enzyme (Osheroff et al., 1994). ICRF-187 binds to Topo II at a stage when religated double-stranded DNA is still locked on the enzyme so that it inhibits enzymatic activity. Due to the importance of this enzyme in cell cycle progression, inhibition of the enzymatic activity by catalytic Topo II inhibitors leads to a block of cell cycle progression at G2/M. Because this portion of the drug-bound enzyme no longer enters the catalytic cycle, the drug reduces the amount of active Topo II required for formation of new DNA-protein complexes targeted by complex-stabilizing inhibitors such as VM-26. Therefore, ICRF-187 treatment may make less target available for complex-stabilizing agents, thereby leading to less DNA damage. From the clinical perspective, inhibition of VM-26-induced FasL expression by ICRF-187 raises the possibility that FasL expression can be modulated by these drug combinations.

Although Topo II inhibitors induce FasL expression, whether such induction of FasL plays a role in drug-induced apoptosis is controversial (Friesen et al., 1996; Eischen et al., 1997; Fulda et al., 1997; Villunger et al., 1997). However, another aspect of drug-induced FasL expression is its impact on the immune system. FasL induced by anticancer drugs has been shown to be functional; it can kill T cells in vitro (Strand et al., 1996). If this type of FasL induction occurs in vivo, it would imply that surviving tumor cells could use FasL as a weapon against T lymphocytes after drug treatment. This may be more likely the case for those tumors that lack Fas expression. Support for this hypothesis comes from our preliminary results showing that the induction of FasL is independent of Fas status; in other words, Topo II inhibitors can induce FasL expression in Fas-deficient cells (Y.-Y. Mo and W. T. Beck, unpublished data). Therefore, under such conditions, the induction of FasL is a "side effect" of the antitumor drug. Melanomas in some patients express elevated FasL (Hahne et al., 1996), and up-regulation of FasL has also been observed in human lung carcinoma (Niehans et al., 1997). Mechanisms of FasL up-regulation in these tumor cells are not clear, but it could be a consequence of exposure to anticancer agents or irradiation that cancer patients have usually received for therapy. Moreover, UV irradiation has been shown to induce FasL expression at least in two cases (Leverkus et al., 1997; Gutierrez-Steil et al., 1998).

Our results suggest that DNA damage caused by complex-forming Topo II inhibitors triggers FasL induction, but little is known about factors or intermediate events that link DNA damage and FasL induction; evidence suggests that activation of nuclear factor κB and JNK pathways are involved in the induction of FasL (Kasibhatla et al., 1998). In addition, DNA-PK has been implicated in modulating induction of p53

by phosphorylation in response to DNA damage and thus impairing the ability of MDM2 to inhibit p53-dependent transactivation (Shieh et al., 1997). Understanding these events and factors will provide insight into the signaling pathway that stimulates FasL expression in response to DNA-damaging agents.

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EXHIBIT C

Induction of Sensitivity to Doxorubicin and Etoposide by Transfection of MCF-7 Breast Cancer Cells with Heregulin β-2[1]

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ABSTRACT

***HER2* (*erb*B-2) proto-oncogene amplification and/or overexpression correlate with poor prognosis in many malignancies. The precise biological role of this oncogenic signaling pathway (which also involves the *HER4* gene) in breast cancer is unclear. One property conferred by this oncogene relates to response to drug therapy. Clinical studies support an association between *HER2* overexpression and resistance to alkylating agents (cisplatinum and cyclophosphamide). Data from the Cancer and Leukemia Group B 8869/8541 study indicate enhanced dose responsiveness to doxorubicin (Adriamycin) in patients who overexpress the *HER2* receptor.**

Heregulin β-2, a naturally occurring ligand that activates the *HER2* receptor by inducing its heterodimerization with the *HER4* receptor, has recently been cloned. The ability of this ligand to phosphorylate the *HER2* receptor exogenously allows us to study the effect of *HER2* activation on cancer cell behavior. To study the relationship between chemotherapy response and activation of *HER2*, MCF-7 cells expressing biologically active heregulin were assessed for response to doxorubicin and etoposide, both of which are topoisomerase IIα (topo IIα) inhibitors. Several clones show markedly increased sensitivity to these drugs. In addition, the same wild-type MCF-7 cells transfected with heregulin β-2 under the control of an inducible promoter also show this dose-response relationship to doxorubicin after the expression of heregulin β-2 is activated by zinc. The modulation of topo IIα was studied in the cell lines transfected with heregulin. topo IIα mRNA and protein (total protein and enzymatic decatenating activity) were found to be up-regulated in heregulin β-2-transfected cells. Moreover, topo IIα promoter activity was also modestly increased in heregulin β-2-transfected cells. Because up-regulation of topo IIα *in vitro* and in clinical specimens is associated with increased response to doxorubicin (presumptively by an increase in drug substrate), this may be the mechanism of the increased sensitivity to doxorubicin seen in heregulin β-2-transfected cells. This implies that activation of *HER2* or one of the other members of the receptor family may increase sensitivity to doxorubicin by up-regulation of topo IIα.

This finding suggests the use of receptor/ligand expression to direct patient-specific therapeutic choices (*e.g.*, doxorubicin *versus* alkylator-based regimens) and the use of biological agents (such as heregulin) in combination with certain chemotherapeutic agents to enhance response to treatment in breast cancer patients.

INTRODUCTION

Oncogene activation has been studied in an attempt to define a molecular correlation for the clinical behavior of breast cancer (1). Numerous studies have indicated a role for the *HER2* oncogene in breast cancer and its correlation with poor prognosis (2, 3). *HER2* is overexpressed in nearly 30% of human breast cancer specimens and is associated with poor outcome in most studies, particularly in node-positive patients.

Several possible ligands that modulate p185*HER2* signal transduction in human breast cancer cells or NIH-3T3 cells overexpressing *HER2* have been characterized. These include the 45-kDa human heregulin (4), a 25-kDa neu/erbB-2 ligand growth factor from bovine kidney (5), the rat Neu differentiation factor (6), and the molecule gp30 (7, 8). Heregulin β-2 has recently been cloned and shown to activate the *HER2* receptor through heterodimerization with the *HER4* receptor (4, 9). Hence, this molecule can induce phosphorylation of the *HER2* receptor without requiring receptor overexpression. Because receptor overexpression and homodimerization are difficult to manipulate *in vitro*, activation of the *HER2* receptor has been studied by construction of *HER2*-epidermal growth factor receptor chimeras activated by epidermal growth factor, a situation that is somewhat artificial. Using heregulin β-2, the effects of *HER2* activation can be studied *in vitro* and *in vivo* using ligand activation.

The relationship between *HER2* receptor overexpression and drug sensitivity is of considerable interest, because this may allow a better prediction of response to chemotherapy. *HER2* activation has been associated with tumor cell resistance to several cytotoxic compounds, including the chemotherapy agent cisplatinum (10), tumor necrosis factor (11), the action of natural killer cells (12), and tamoxifen (13, 14) on breast cancer

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cells. However, increased response to anthracycline (*e.g.*, doxorubicin)-containing treatment has also been correlated with *HER2* overexpression (15, 16). Further understanding of the molecular mechanisms behind this differential sensitivity may lead to the selection of optimal chemotherapy in these otherwise bad-prognosis tumors.

It is thought that doxorubicin acts by binding to topo IIα.[3] topo IIα is a DNA-modifying enzyme that binds to the double helix to release torsional stress and create double-strand breaks that allow replication to occur. Drugs that interfere with topo IIα include the anthracyclines (doxorubicin and daunorubicin), VP-16, teniposide, and amascarine. These agents seem to act by binding covalently with topo IIα after double-strand breaks have occurred and induce lethal cellular damage. Increased topo IIα expression is associated with sensitivity to these agents, both in cell lines and tumors, presumably due to the increased substrate on which the drug may act (17, 18). topo IIα, which occurs in the same amplicon on chromosome 17 as *HER2*, has been shown to be altered by amplification, point mutation, and deletion in breast cancers that overexpress *HER2* (19). A recent study demonstrated that increased expression of topo IIα is associated with c-*erb*B-2 (*HER2*) overexpression in breast cancer (20). Therefore, it seems that topo IIα may be commonly overexpressed with this oncogene, leading to a mechanism for increase in sensitivity to topo IIα inhibitors.

Although clinical studies imply a dose-response relationship between receptor overexpression and response to anthracycline treatment, studying this phenomenon *in vitro* has been problematic. Cell lines that overexpress *HER2* have been examined for differences in drug sensitivity/resistance with varying results. The ability to modulate *HER2* activation using heregulin β-2, along with the unelucidated relationship between *HER2* overexpression and response to doxorubicin, led us to explore heregulin β-2-mediated activation of the *HER2* receptor as it relates to drug sensitivity. To study whether activation of *HER2* would modulate drug responsiveness, we examined breast cancer cells transfected with heregulin β-2 cDNA. MCF-7 breast cancer cells expressing biologically active heregulin β-2 (33) were assessed for response to doxorubicin and VP-16, both of which are topo IIα-inhibitory agents. It seems, based on our *in vitro* data, that activation of the *HER2/HER4* receptors using heregulin β-2 induces sensitivity to doxorubicin through upregulation of topo IIα. It is also possible that heregulin itself, in the absence of receptor activation, may induce sensitivity to topo IIα inhibition through an as yet unknown mechanism.

MATERIALS AND METHODS

Cell Lines. MCF-7 (American Type Culture Collection) breast cancer cells were transfected with a heregulin construct (heregulin β-2 cDNA, amino acids 1–426, was inserted into the pRc/CMV eukaryotic expression vector under the control of cytomegalovirus early gene promoter) with subsequent selection of stable pooled (T4 and T5) and single-clone (T6, T7, and S2) populations (33).[4] These MCF-7/T clones were used for all experiments, except as indicated. In addition, vector-transfected cells (MCF-7/V) and MCF-7/WT cells were used as controls. MCF-7/ADR cells (Ken Cowan, NIH, Bethesda, MD) were used as a doxorubicin-resistant cell line. MCF-7/heregulin-MT cells were created by transfection of MCF-7 cells with heregulin β-2 under the control of a zinc-inducible MT promoter. Cells were stably transfected and selected as described above.[4] Characterization of cell lines as high, medium, or low expressors refers to the mRNA levels of heregulin β-2 after induction with zinc chloride (100 μM) for 2 days and measurement by RNase protection (data not shown). In addition, the antisense sequence of heregulin β-2 under control of the MT promoter was transfected into parental MCF-7 cells as a control. The antisense clone had no detectable levels of heregulin β-2 mRNA.

Anchorage-dependent Cytotoxicity Assays. MCF-7/WT, MCF-7/ADR, MCF-7/T, and MCF-7/V cells were plated at 1000–2000 cells/well in IMEM + 10% FCS in a 96-well microtiter dish on day 0. Cells were treated with increasing concentrations of doxorubicin (0.001–10 μM) or VP-16 (0.1–1000 μM) with continuous exposure and assayed for viability on day 7 (at confluence of control sample). Cell viability was assessed by XTT assay (21). Specifically, XTT (Polysciences, Inc.) solution was prepared at 1.0 mg/ml in prewarmed IMEM-phenol Red-free media, incubated for 20 min, and incubated with 10 μl of phenazine methosulfate solution (1.53 mg/ml) per milliliter of XTT solution for 20 min. This solution (50 μl) was added to 200 μl of IMEM + phenol Red-free media in microtiter plates containing treated cells. The plates were incubated at 37°C for 4 h, shaken for 20 min, and read at 450 nm on an ELISA plate reader. Similar assays were performed for MCF-7/MT cells that were incubated before plating in 100 μM $ZnCl_2$ or serum-containing media alone.

Anchorage-independent Cytotoxicity Assays. A liquid overlay culture system was used to generate three-dimensional cultures. Briefly, each well of a 24-well plate was coated with 250 μl of 1% Seaplaque-agarose in serum-free medium (IMEM). Cells (100,000) in complete medium were added to each well of a 24-well dish and incubated at 37°C. Approximately 4 days in culture were required to allow the formation of spheroid structures for all cell lines. Triplicate wells of spheroids were subsequently incubated with 1 ml of increasing dilutions of doxorubicin (0.001–10 μM). After 5 days under those conditions, spheroids were removed, washed three times, dispersed, and plated in triplicate in 100-mm tissue culture dishes for colony-counting assays (22).

Immunoprecipitation and Western Blot Analysis for topo IIα Protein. Nuclear protein extracts were prepared from MCF-7/WT and MCF-7/T cells. After preclearing the extracts with protein A-Sepharose, samples were immunopre-

[3] The abbreviations used are: topo IIα, topoisomerase IIα; VP-16, etoposide; MT, metallothionine; WT, wild-type; IMEM, Iscove's MEM; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; CAT, chloramphenicol acetyltransferase; MDR, multidrug resistance.

[4] C. Tang, M. Cardillo, D. Yang, C. Cho, C. Waibel, C. Perez, and R. Lupu. Heregulin β-2 isoform can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice, submitted for publication.

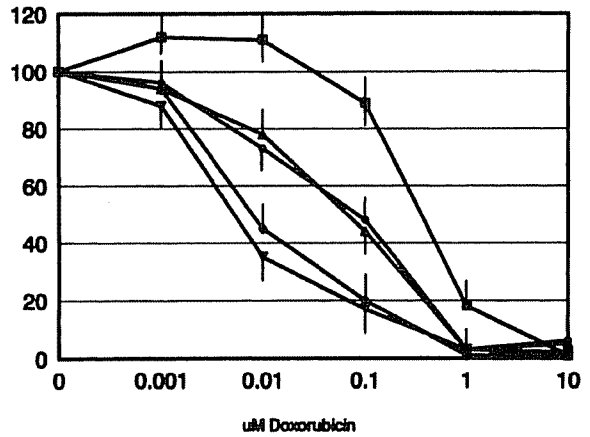

Fig. 1 Heregulin-transfected cells show increased sensitivity to doxorubicin in anchorage-dependent cytotoxicity assays. MCF-7/WT (●), MCF-7/ADR (■), MCF-7/T (▼, MCF-7/T5; ◆, MCF-7/T6), and MCF-7/V (▲) cells were plated at 1000–2000 cells/well in IMEM + 10% FCS in a 96-well microtiter dish on day 0. Cells were treated with increasing concentrations of doxorubicin (0.001–10 μM) with continuous exposure starting on day 1 and assayed for viability on day 7 (at confluence of the control sample). The plates were incubated at 37°C for 4 h and shaken for 20 min, and absorbance was measured at 450 nm on an ELISA plate reader. Cytotoxicity is expressed as the percentage of control of absorbance; *points*, means of quadruplicate wells; *error bars*, SD.

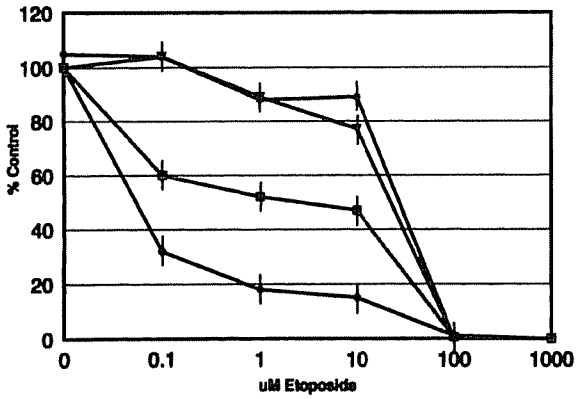

Fig. 2 Heregulin-transfected cells show increased sensitivity to VP-16 in anchorage-dependent cytotoxicity assays. MCF-7/WT (●), MCF-7/T (■, MCF-7/T5; ◆, MCF-7/T6), and MCF-7/V (▼) cells were plated at 1000–2000 cells/well in IMEM + 10% FCS in a 96-well microtiter dish on day 0. Cells were treated with increasing concentrations of VP-16 (0.1–1000 μM) starting on day 1 with continuous exposure and assayed for viability on day 7 (at confluence of the control sample). The plates were incubated at 37°C for 4 h and shaken for 20 min, and the absorbance was measured at 450 nm on an ELISA plate reader. Cytotoxicity is expressed as a percentage of control of absorbance; *points*, means of quadruplicate wells; *error bars*, SD.

cipitated with an anti-p170 human topo IIα rabbit polyclonal antibody (1 unit/ml; TopoGEN, Inc., Columbus, OH). After incubation on ice, the antigen-antibody complex was precipitated with 10% protein A-Sepharose, washed, and separated using 4–20% SDS-PAGE. Purified p170 topo IIα was used as a marker. Protein samples were transferred onto nitrocellulose using the Western blot technique. After incubation with a topo II mouse monoclonal antibody (1 unit/ml), blots were visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

Northern Blot Analysis. mRNA was extracted from MCF-7/V and MCF-7/T cells in log growth phase using the RNazol (Teltest, Inc., Friendswood, TX) method. mRNA (20 μg) was run on a 1% agarose/formaldehyde gel by electrophoresis. Intact 18S and 28S bands were seen by ethidium bromide staining; RNA was subjected to Northern blot analysis. The blot was hybridized with a random-primed [^{32}P]dCTP-labeled segment of the COOH-terminal fragment of topo II cDNA (topo-Z2) kindly provided by Dr. Leroy Liu (UMDNJ R. W. Johnson Medical School, Piscataway, NJ). After extensive washes under standard conditions (23), bands were visualized by autoradiography. Loading and transfer efficiency was normalized with a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, under the same conditions.

Transient Transfection and CAT Assay. A pCAT BASIC construct containing a 2400-bp promoter sequence for the topo IIα gene linked to a chloramphenicol acyltransferase reporter was used for transient transfections. The CAT-topo II construct, kindly provided by Dr. Ian Hickson (Imperial Cancer Research Fund, Oxford, England), was transfected into MCF-7/WT, MCF-7/V, and MCF-7/T cell lines at 80% confluence to control for cell cycle changes. Calcium phosphate precipitation-type transfection was performed. Specifically, plasmid was incubated for 15–30 min in a $CaCl_2$-containing solution to which phosphate was added. Cells were then washed and incubated with the calcium phosphate-plasmid solution for 15–24 h at 37°C. The cells were subsequently washed several times with PBS and incubated for an additional 48 h. Cells were harvested, lysed, and incubated with [^{14}C]chloramphenicol in the presence of acetyl-CoA. CAT activity was measured on TLC plates by exposure to X-ray film and autoradiography. Quantitation of CAT activity was performed by densitometry. CAT activity was expressed as the amount of acetylated CAT:total CAT × transfection rate (from Hirt assay).

Hirt Assay (CAT Transfection Rate). Transfection assays were normalized by analysis of topo II plasmid DNA content in transfected cells using the Hirt assay. This method uses extraction of DNA followed by Southern analysis (24) and subsequent measurement of the signal by densitometry. Briefly, transfected cells were harvested and resuspended in Tris-EDTA buffer, and DNA was extracted with SDS and NaCl on ice for 16 h. DNA quantity was then normalized by spectrophotometry, and equal amounts were digested with *Bam*HI. DNA was separated by gel electrophoresis and blotted onto nitrocellulose. Subsequently, the topo II CAT gene was radiolabeled with [^{32}P]dCTP and used to probe the blot. Hybridized blots were developed by autoradiography, and measurement of the 6.7-kb band was assessed by densitometry to assess for the copy number of the plasmid.

RESULTS

Constitutive Activation of *HER2* and *HER4*. Heregulin β-2-transfected cells secrete biologically active ligand and

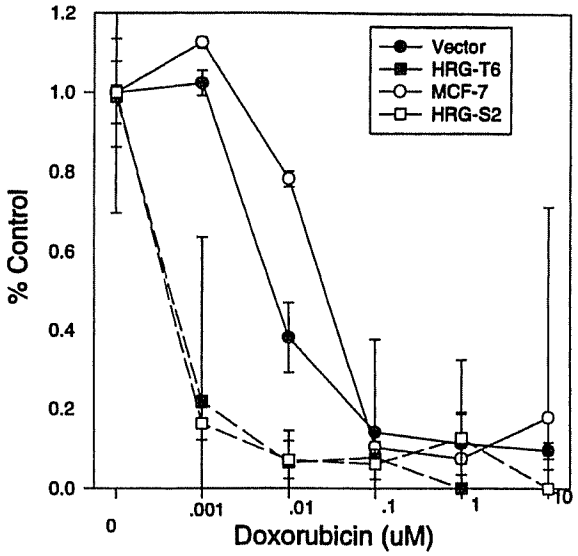

Fig. 3 Heregulin-transfected cells show increased sensitivity to doxorubicin in anchorage-independent cytotoxicity assays. A liquid overlay culture system was used to generate three-dimensional cultures. Briefly, each well of a 24-well plate was coated with 250 μl of 1% Seaplaque-agarose in serum-free medium (IMEM). Cells (100,000) in complete medium were added to each well and incubated at 37°C in a 5% CO_2 incubator. After the formation of spheroids (approximately 4 days in culture), triplicate wells were treated with 1 ml of increasing dilutions of doxorubicin (0.001–10 μM). After 5 days under those conditions, the spheroids were removed, washed three times, dispersed, and plated in triplicate in 100-mm tissue culture dishes for colony-counting assays (25).

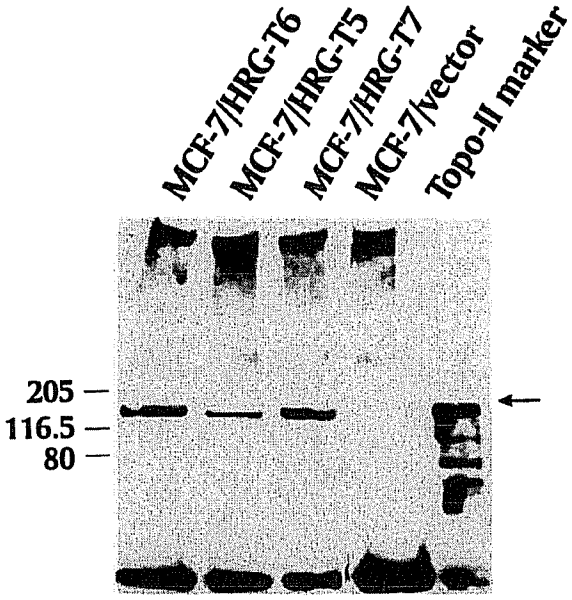

Fig. 4 topo II protein levels are found to be increased in those MCF-7/T cells that had increased sensitivity to topo II-inhibitory agents. Nuclear protein extracts were prepared from MCF-7/WT and MCF-7/T cells. Equal amounts of nuclear extract, based on protein concentration, were precleared and incubated with protein A-Sepharose. Subsequently, samples were immunoprecipitated with an anti-p170 human topo II anti-rabbit polyclonal antibody (1 unit/ml; TopoGEN, Inc.). After incubation on ice, the antigen-antibody complex was precipitated with 10% protein A-Sepharose, washed, and separated using 4–20% SDS-PAGE. Purified p170 topo II was used as a marker (TopoGEN, Inc.). Protein samples were transferred onto nitrocellulose using the Western blot technique. After incubation with a topo II anti-mouse monoclonal antibody (1 unit/ml), blots were visualized using enhanced chemiluminescence (Amersham). The lower 50-kDa band, which is constant in all wells, represents the γ-globulin heavy chain.

have constitutively activated *HER2* and *HER4*.[4] These cells were used because they show several features of breast cancer progression, including loss of sensitivity to antiestrogens and ability to grow in anchorage-independent assays and form tumors in nude mice in the absence of estrogen. Immunoblotting with antiphosphotyrosine after immunoprecipitation with *HER2*, *HER3*, and *HER4* revealed that heregulin β-2-transfected cells have an increase in phosphorylation of all three receptors, implying activation of *HER2*, *HER3*, and *HER4* (33). Of note, pooled populations (MCF-7/T4 and MCF-7/T5) had lower levels of *HER2*, *HER3*, and *HER4* phosphorylation than single-clone populations (MCF-7/T6, MCF-7/T7, and MCF-7/S2).

Increased Sensitivity to Doxorubicin and VP-16 in Anchorage-dependent Cytotoxicity Assays. Heregulin β-2-transfected cells were assessed for response to cytotoxic drugs, because the phenotype of drug resistance is important for understanding the biology of heregulin β-2 activation and cancer progression. To test drug sensitivity, cytotoxicity assays were performed. Heregulin β-2-transfected (MCF-7/T) cells were plated at a density that would allow confluence of the control sample at day 7. After allowing 24 h for adherence to plates, cells were treated with increasing concentrations of doxorubicin and cytotoxicity, measured using a XTT cell viability assay on day 7. MCF-7/T cells showed an 11-fold increase in sensitivity to doxorubicin (LD_{50} = 0.009 μM) as compared to MCF-7/WT and MCF-7/V cells (LD_{50} = 0.1 μM) and were 88-fold more sensitive than MCF-7/ADR cells (LD_{50} = 0.8 μM), a multidrug-resistant cell line. As shown in Fig. 1, this phenotype was seen in several transfected clones with high levels of constitutive *HER2*, *HER3*, and *HER4* phosphorylation (MCF-7/T6, MCF-7/T7, and MCF-7/S2) but was not seen consistently in the pooled populations of MCF-7/T cells with low-to-intermediate levels of phosphorylation (MCF-7/T4 and MCF-7/T5). We felt that this was due to the variable expression of heregulin β-2 in pooled populations after several passages.

We tested the sensitivity of heregulin β-2-transfected cells to VP-16, another topo IIα inhibitor, to determine whether the phenotype was generalizable. Cytotoxicity assays were performed as described above with increasing concentrations of VP-16. Again, an increase in sensitivity to VP-16 (LD_{50} = 0.8 μM) was seen in transfected cells compared to vector control (LD_{50} = 6 μM; Fig. 2). No difference was seen in drug sensitivity to cisplatinum, 5-fluorouracil, or Taxol (drugs that do not cause cytotoxicity through topo II) when heregulin β-2-transfected cells were compared to WT and vector (data not shown) in anchorage-dependent assays. However, preliminary data suggest that three of the clones are 2–3-fold more resistant to

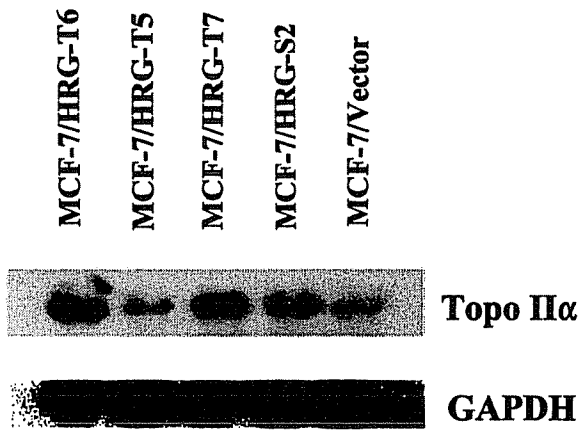

Fig. 5 topo II mRNA levels were higher in transfected cells as compared to vector-transfected cells. mRNA was extracted from MCF-7/V and MCF-7/T cells in log growth phase using the RNazol (Teltest Inc.) method. mRNA (20 μg) was run on a 1% agarose/formaldehyde gel by electrophoresis. Intact 18S and 28S bands were seen by ethidium bromide staining; RNA was subjected to Northern blot analysis (1). The blot was hybridized with a random-primed [^{32}P]dCTP-labeled segment of the carboxyl-terminal fragment of topo II cDNA (topo-Z2). After extensive washing, the bands were visualized by autoradiography. Loading and transfer efficiency were normalized to a housekeeping gene (*GAPDH*) under the same conditions.

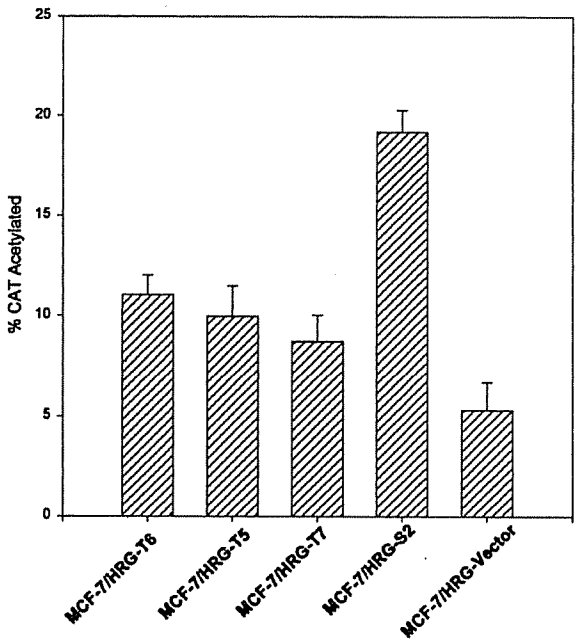

Fig. 6 MCF-7/T cells were shown to have a 1.5–4-fold increase in topo II promoter activity as compared to MCF-7/V cells when normalized to the transfection rate by Hirt assay. A pCAT BASIC construct containing a 2400-bp promoter sequence for the topo IIα gene, linked to a chloramphenicol acyltransferase reporter, was used for transient transfections. The CAT-topo II construct was transfected into MCF-7/WT, MCF-7/V, and MCF-7/T cell lines at 80% confluence. Calcium phosphate precipitation-type transfection was performed. Cells were harvested, lysed, and incubated with [^{14}C]chloramphenicol in the presence of acetyl-CoA. CAT activity was measured on TLC plates by exposure to X-ray film and autoradiography. Quantitation of CAT activity was performed by densitometry. CAT activity was expressed as the amount of acetylated CAT:total CAT × the transfection rate (from the Hirt assay). SDs were <2% CAT acetylated over an average of three samples.

cisplatinum in soft agar cloning assays (25). The mechanism of this finding is currently under investigation.

Increased Sensitivity to Doxorubicin in Anchorage-independent Cytotoxicity Assays. To test whether sensitivity to topo II inhibitors occurred in an anchorage-independent setting, spheroid cultures of heregulin β-2-transfected cells were exposed to increasing doses of doxorubicin. The spheroid method was used because it more closely approaches the solid tumor model of drug resistance/sensitivity and therefore the *in vivo* situation (22). A liquid overlay culture system was used, and 100,000 cells in complete medium were added to each well of a 24-well dish and incubated at 37°C. Approximately 4 days in culture were required to allow the formation of spheroid structures for all cell lines. The size and number of spheroid colonies formed did not differ significantly between any of the heregulin β-2-transfected or vector-transfected cells. Triplicate wells of spheroids were exposed to increasing doses of doxorubicin and allowed to grow for 5 days under those conditions. Subsequently, spheroids were washed, disaggregated, and plated in 100-mm tissue culture for colony-counting viability assays. Again, MCF-7/T cells were 10–100 times more sensitive to doxorubicin than were the controls (LD_{50} = 0.0008 *versus* 0.008–0.08 μM; Fig. 3).

topo IIα is Up-Regulated in Heregulin β-2-transfected Cells at the Level of Protein, mRNA, and Promoter. To determine whether increased sensitivity to doxorubicin and VP-16 was mediated by alterations in topo IIα activity, we examined topo II expression in heregulin β-2-transfected cells and controls. topo II protein levels were measured by immunoprecipitation and Western blot and found to be increased in those MCF-7/T cells that had increased sensitivity to topo II-inhibitory agents. Fig. 4 is representative of four separate experiments in which topo IIα protein was expressed at between 5- and 10-fold higher levels in heregulin β-2-transfected cells compared with vector-transfected cells. Some expression of topo IIα was also seen in vector-transfected cells, but the relatively lower levels were not always immunoprecipitated as efficiently as in the heregulin β-2-transfected cells.

To assess whether topo IIα up-regulation was altered at the level of the mRNA, we performed Northern blot experiments using a cDNA probe against the topo IIα carboxyl terminus. Results show that topo II mRNA levels were 1.4–5.8 times higher in transfected cells as compared to those in vector, except in pooled clone MCF-7/T5 (Fig. 5). As previously mentioned, this finding is consistent with the variability in drug sensitivity seen with pooled clones. Of note, expression of topo II mRNA was still seen in vector-transfected cells, whereas topo IIα protein was undetectable. This implies either further posttranscriptional modification of the topo IIα message in MCF-7/T cells or that protein levels in the vector control were below the level of detection sensitivity.

Given that heregulin β-2 transfectants showed an up-reg-

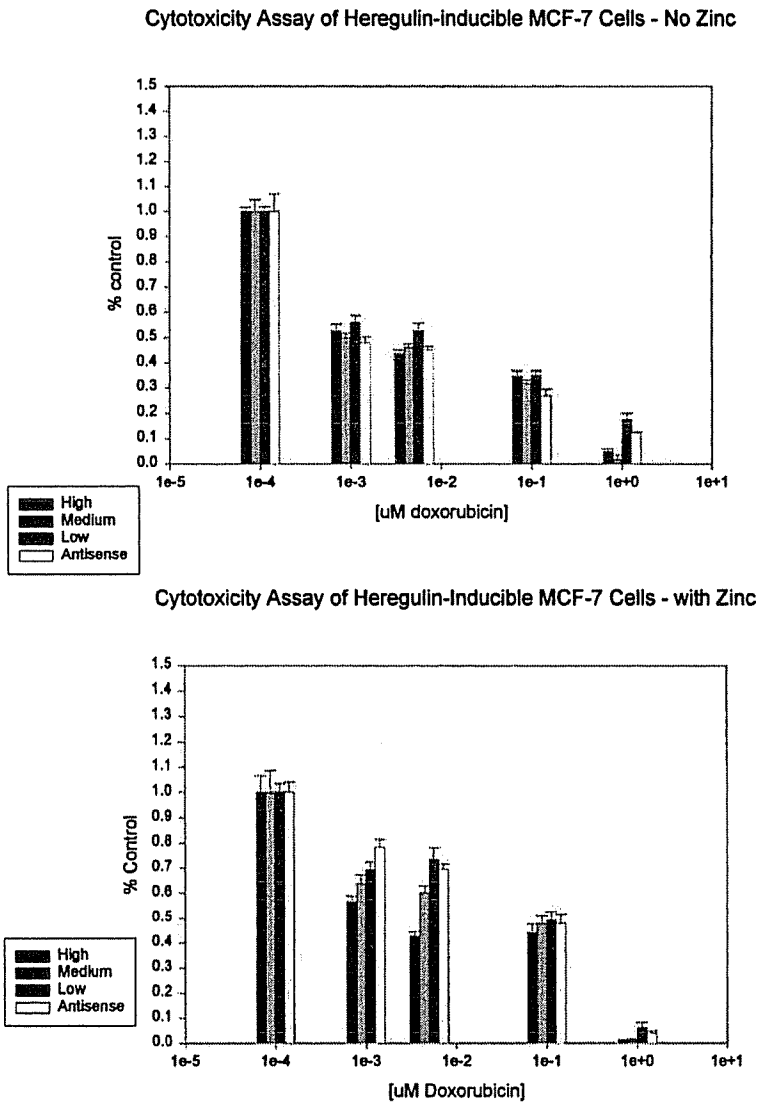

Fig. 7 MCF-7 cells transfected with heregulin β-2 under the control of a MT-inducible promoter have increased sensitivity to doxorubicin. Characterization of cell lines as high, medium, or low expressors refers to the mRNA levels of heregulin β-2 after induction with zinc chloride and measurement by RNase protection. MCF-7/heregulin-MT sense and antisense transfectants were plated 1000–2000 cells/well in IMEM + 10% FCS in a 96-well microtiter dish on day 0, ± induction with $ZnCl_2$, 100 μM × 2 days. Cells were treated with increasing concentrations of doxorubicin (0.001–10 μM) starting on day 1 with continuous exposure and assayed for viability on day 5. Cell viability was assessed by XTT assay.

ulation of topo IIα mRNA and protein, we decided to examine the promoter activity of the topo II gene to determine whether increased transcriptional activity could account for this observation. topo IIα promoter constructs linked to a CAT reporter gene were transiently transfected into heregulin β-2-transfected cells and controls. As shown in Fig. 6, MCF-7/T cells were shown to have a 1.5–4-fold increase in topo IIα promoter activity as compared to MCF-7/V cells when normalized to transfection rate by Hirt assay. Although modest, this is statistically significant and may account, at least in part, for the increased activity noted in MCF-7/T cells.

Up-Regulation of topo IIα Does Not Seem To Be Due to Changes in Cell Cycle Distribution in Heregulin β-2-transfected Cells. topo IIα levels have been shown to be cell cycle dependent (27). Therefore, to determine whether increased levels of topo IIα was due to altered distribution of cell cycle, cells were harvested at equal time points and cell cycle distribution measured. No difference in cell cycle distribution was seen between clones or their vector (data not shown). In addition, the doubling time of MCF-7/T, MCF-7/V, and MCF-7/WT is similar in serum-containing conditions.

Drug Sensitivity Is Not Due to Alterations in MDR Expression. The p170 glycoprotein product of the MDR gene has been shown to lead to increased resistance to doxorubicin and other drugs. To assess if expression of the *mdr* gene was altered in the heregulin β-2-transfected, leading to increased sensitivity, Western blot for p170 glycoprotein was performed. Expression of this protein was not significantly changed in heregulin-transfected cells as compared with controls (data not shown). With the recent discovery of the MDR-related protein MRP, it would be useful to examine these cell lines for this alternate drug-resistance mechanism (27).

Drug Sensitivity Can Be Induced in MCF-7 Cells Transfected with Heregulin β-2 under the Control of an Inducible Promoter. To determine whether the drug sensitivity of these cells was directly related to the expression of heregulin, MCF-7 cells transfected with heregulin-β-2 linked to a MT-inducible promoter were used to assess drug sensitivity. MCF-7/MT cells were treated with and without zinc chloride (100 μM) for 2 days to induce heregulin β-2 production and then exposed to increasing concentrations of doxorubicin. Preliminary experiments with MT-inducible cells show a dose-response effect to doxorubicin, with increasing levels of heregulin β-2 expression corresponding to increased sensitivity to doxorubicin (Fig. 7). This suggests that the expression of heregulin β-2 is correlated with the induction of doxorubicin sensitivity in these cells.

DISCUSSION

The current study demonstrates that heregulin β-2-transfected cells showed a marked increase in sensitivity to topo IIα-inhibitory drugs compared to WT and vector controls. This effect was also observed in cells transfected with heregulin linked to an inducible promoter with increasing levels of induction correlating with increasing sensitivity to doxorubicin. Furthermore, when expression of topo IIα was studied, increased expression was observed at the both the protein and mRNA level in MCF-7/heregulin β-2-transfected cells.

topo IIα is well known for its role in mediating doxorubicin cytotoxicity and has been increasingly shown to have a role in doxorubicin resistance in solid tumors (28, 29). It seems that increased expression of topo IIα is associated with sensitivity to doxorubicin both in cell lines and in clinical specimens. In tumors from breast cancer patients, 83.3% of responsive tumors expressed high levels of the enzyme. It was also shown that nine of nine tumors resistant to doxorubicin expressed undetectable or low levels of topo IIα (18). In addition, numerous studies demonstrate the relationship between decreased topo IIα activity and resistance to topo IIα inhibitors (27, 28). Based on our observation that topo IIα is consistently elevated in the heregulin β-2-transfectants that are sensitive to doxorubicin, this may be the mechanism of increased sensitivity. In addition, we did not see a change in the p170 glycoprotein responsible for the multidrug-resistant phenotype (30) in the heregulin β-2-transfected cell lines that would explain this phenotype.

Whereas clinical studies support an association between *HER2* receptor overexpression and resistance to alkylating agents (15), data from the Cancer and Leukemia Group B 8869/8541 study indicate a dose-response relationship of doxorubicin in *HER2*-overexpressing patients (16). Specifically, it has been shown that tumors that overexpress *HER2* were more effectively treated with higher doses of doxorubicin-containing FAC (5-fluorouracil, Adriamycin, and cyclophosphamide). Whether this represents sensitivity or resistance to doxorubicin is undetermined. The situation may be that *HER2*-overexpressing tumors are on the linear phase of the dose-response curve, whereas nonoverexpressing tumors are on the plateau phase and therefore achieve little, if any, benefit from doxorubicin.

Heregulin is known to activate the *HER2* receptor by heterodimerization through *HER3* and *HER4*. Based on our studies, it seems that breast cancers that have *HER2* activated are relatively more sensitive to doxorubicin. This phenomenon would seem to be due to the induction of topo IIα in these cells and may explain the mechanism of the dose-response effect seen *in vivo*. It may be that activation of the *HER3* and *HER4* receptor is partly or totally responsible for this change in phenotype; however, no clinical correlations have been performed as yet.

Other investigators have shown resistance to cisplatinum in breast cancer cell lines transfected with *HER2* (31). This is postulated to be due to an increase in DNA excision-repair in *HER2*-overexpressing cells as suggested by the work of Arteaga *et al.* (32) in which reversal of this phenotype was seen using antibodies to *HER2* with a concomitant decrease in the cells' ability to repair DNA adducts. Preliminary experiments performed in collaboration with Dr. Paul Andrew (Department of Pharmacology, Georgetown University, Washington, D.C.) suggest that the heregulin β-2-transfected cells are approximately 2–3-fold more resistant to cisplatinum than are the controls. This suggests a differential sensitivity between doxorubicin (and other topo IIα inhibitors) and alkylators (like cisplatinum) in cells with activation of *HER2*, either by ligand or by receptor overexpression. The potential application of this finding for therapy is the use of *HER2* or heregulin β-2 as a marker for drug response. If breast tumors with activation of *HER2* are more likely to be sensitive to doxorubicin than alkylators, then drug regimens containing this drug as its major active component (*e.g.*, FAC, AC) should be chosen for these patients as opposed to more conventional cyclophosphamide, methotrexate, and fluorouracil regimens.

We are entering an era in which basic research and clinical medicine is being united by molecularly directed therapeutic decisions. Whereas newer agents directed at molecular targets are promising, they have yet to show benefit over conventional drugs. Further experimentation in this area is critical to fully understand the mechanism of response to conventional agents. This will not only allow us to use the tools we have more effectively but will also suggest the optimum ways to combine newer biological agents with older drugs.

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